Development and growth of skeletal muscle

Neil Charles Stickland

DSc

The University of Edinburgh

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Declaration

I hereby declare that none of the publications listed herein have been submitted for any other degree or diploma except that parts of the following publications have been included in a PhD submission to the University of Hull in 1973:

Stickland, N.C. and Goldspink, G. (1973) A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. Anim. Prod. 16, 135-146.

Stickland, N.C. and Goldspink, G. (1975) A note on porcine skeletal muscle parameters and their possible use in early progeny testing. Anim. Prod. 21, 93-95.

Stickland, N. C., Widdowson, Elsie M. and Goldspink, G. (1975) Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscle of pigs. Brit. J. Nutr. 34, 421-428.

Stickland & Goldspink (1978) Number of fibres in the skeletal muscle of miniature pigs. J. Agric. Sci. 91, 255-256.

I hereby declare that, for all publications where I am first author, the practical work and writing up of papers was largely my own responsibility. Where I am not first author the practical work and writing up was shared with the other authors. However, all works, apart from the four listed above, were carried out in my own laboratory and were initiated and lead by myself as the research group leader.

Signed N C Stickland

Date 11 June '97

Abstract

Development and Growth of Skeletal Muscle

The main body of this work contributes to an understanding of the development and growth of skeletal muscle in a range of Vertebrates from fish to pigs. Particular emphasis is paid to the contribution of numbers and types of muscle fibres to overall muscle growth and ultimate mass, and also to the mechanisms whereby factors such as nutrition in mammals and temperature in fish may affect these parameters. The work is divided into three main sections.

The first section covers aspects of prenatal mammalian development including myogenesis and placentation. Muscle develops as two populations of muscle fibres. Primary myofibres form first and this is followed by the formation of a larger population of secondary fibres. Restricting maternal nutrition may compromise the formation of secondary fibres but not primaries. Studies on the placenta and on levels of specific factors, e.g. insulin-like growth factors, has given some insight into the mechanism of nutritional effects on muscle fibre development. Nutritional experiments have highlighted energy levels in the earlier stages of gestation as most critical in the development of muscle fibre number. This finding has been developed in pig experiments which have shown that extra feed in early gestation can produce piglets with more secondary fibres at birth and which grow faster and more efficiently to slaughter.

The second section incorporates work on postnatal mammalian muscle. Studies, on pigs in particular, have shown that primary fibre number relates more to genotype than does secondary fibre number. Total muscle fibre number correlates with some parameters of carcass leanness and with postnatal growth rate and feed conversion efficiency. The influence of factors such as nutrition, dwarfism, obesity and sex on aspects of muscle growth and muscle fibre types has been studied as well as the functional adaptation of muscle metabolism in different species.

The third section includes work on fish muscle development and growth in a range of species. There is particular emphasis on the role of temperature during embryonic stages on the development of muscle cellularity. In salmonid species higher temperatures have been shown to produce muscle with larger but fewer muscle fibres than at lower temperatures. There are also effects on the expression of specific genes during development. The effect on muscle cellularity may have a consequence for the posthatch growth of the fish when subsequently reared at the same temperature. Further work has suggested that oxygen availability may be compromised at the higher temperature and may be a significant factor in the temperature affect on muscle cellularity. Temperature effects on muscle metabolism have also been demonstrated.

Contents

1. **Prenatal mammalian development including myogenesis and placentation**

   a. ***Original articles***

Stickland, N.C. and Purton, M.D. (1977). A quantitative evaluation of placentome development in Zebu cattle (*Bos indicus*). Anat. Histol. Embryol. 6, 81-86.

Stickland, N.C. (1978). A quantitative study of muscle development in the bovine foetus (*Bos indicus*). Anat. Histol. Embryol. 7, 193-205.

Stickland, N.C. (1981). Muscle development in the human fetus as exemplified by m.sartorius: a quantitative study. J. Anat. 132, (4), 557-579.

Stickland, N.C. (1982). Scanning electron miscroscopy of prenatal muscle development in the mouse. Anat. Embryol. 164, 379-385.

Wigmore, P.M.C. and Stickland, N.C. (1983). Muscle development in large and small pig fetuses. J. Anat. 137, 235-245.

Wigmore, P.M.C. and Stickland, N.C. (1983). DNA, RNA and protein in skeletal muscle of large and small pig fetuses. Growth 47, 67-76.

Wigmore, P.M.C. and Stickland, N.C. (1985). Placental growth in the pig. Anat. Embryol. 173, 263-268.

Ward, S.S. and Stickland, N.C. (1991). Why are slow and fast muscles differentially affected during prenatal undernutrition? Muscle and Nerve 14, 259-267.

Ward, S.S. and Stickland, N.C. (1992). The effect of undernutrition in the early postnatal period on skeletal muscle tissue. Brit. J. Nutr. 69, 141-150.

Dwyer, C.M. and Stickland, N.C. (1992). Does the anatomical location of a muscle affect the influence of undernutrition on muscle fibre number? J. Anat. 181, 373-376.

Dwyer, C.M., Madgwick, A., Crook, A.R. and Stickland, N.C. (1992). The effect of maternal undernutrition on the growth and development of the guinea pig placenta. J. Dev. Physiol. 18, 295-302.

Dwyer, C.M. and Stickland, N.C. (1992). The effect of maternal undernutrition on maternal and fetal serum insulin-like growth factors, thyroid hormones and cortisol in the guinea pig. J. Dev. Physiol. 18, 303-313.

Dwyer, C.M. and Stickland, N.C. (1994). Supplementation of a restricted maternal diet with protein or carbohydrate alone prevents a reduction in fetal muscle fibre number in the guinea pig. Brit. J. Nutr. 72, 173-180.

Dwyer, C.M, Stickland, N.C. and Fletcher, J.M. (1994). The influence of maternal nutrition on muscle fiber number development in the porcine fetus and on subsequent postnatal growth. J. Anim. Sci. 72, 911-917.

Dwyer, C.M., Madgwick, A.J.A., Ward, S.S. and Stickland, N.C. (1995). Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. Reprod. Fertil. Dev. 7, 1285-92.

Published Abstracts

Wigmore, P.M.C. and Stickland, N.C. (1981). Prenatal muscle development in the pig: a comparison of the largest and smallest litter mates. J. Anat. 133, 132. (Proceedings of Meeting April 1981, Cambridge).

Stickland, N.C. (1982). A scanning electron microscopic study of prenatal muscle development *in vivo*. J. Anat. 134, 607-8. (Proceedings of Meeting December 1981, London).

Brown, S.C. and Stickland, N.C. (1987). Backscattered electron imaging of nuclei in prenatal muscle development. J. Anat. 155, 240. (Proceedings of Meeting July 1987, Dundee).

Ward, S.S. and Stickland, N.C. (1987). The effect of undernutrition on fibre number in myogenesis. J. Anat. 155, 240. (Proceedings of Meeting July 1987, Dundee).

Stickland, N.C. (1988). Prenatal muscle development and its effect on postnatal growth. J. Anat. 161, 223. (Invited Symposium presentation at Meeting July 1988, Dublin)

Ward, S.S. and Stickland, N.C. (1988). The effect of undernutrition on the myogenesis of fast and slow twitch muscles of the guinea pig. J. Anat. 161, 226. (Presented at Meeting July 1988, Dublin).

Madgwick, A.J.A. and Stickland, N.C. (1988). The effect of undernutrition on nuclear populations in fast and slow twitch muscles during myogenesis in the guinea pig. J. Anat. 161, 226-227. (Presented at Meeting July 1988, Dublin).

Dwyer, C.M., Madgwick, A., Crook, A.R. and Stickland, N.C. (1993). The effect of maternal undernutrition on the development of placental components in the guinea pig. J. Anat. 183, 186. (Proceedings of Meeting January 1993, London).

Dwyer, C.M., Madgwick, A., Ward, S.S. and Stickland, N.C. (1993). The effect of maternal undernutrition, imposed before or after the first trimester, on muscle fibre development in the guinea pig fetus. J. Anat. 183, 200. (Proceedings of Meeting January 1993, London).

Brown, S.C. and Stickland, N.C. (1996). Muscle development in mice selected for large and small body weight. J. Anat. 189, 230. (Proceedings of Meeting January 1996, London).

Walters, E.H., Stickland, N.C. and Loughna, P.T. (1997). Immunohistochemical localisation of Myo D and myogenin in developing fast and slow muscle fibres of the foetal mouse. Anat. Histol. Embryol. (in press). (Proceedings of XXI EAVA Congress July 1996, Lugo, Spain).

Walters, E.H., Stickland, N.C. and Loughna, P.T. (1997). Distribution of Myo D and myogenin in developing fast and slow muscle fibres of the fetal mouse. J. Anat. (in press). (Proceedings of Meeting December 1996, London).

2. Postnatal mammalian muscle including growth and adaptation

a. *Original articles*

Stickland, N.C. and Goldspink, G. (1973). A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. Anim. Prod. 16, 135-146.

Stickland, N.C. and Goldspink, G. (1975). A note on porcine skeletal muscle parameters and their possible use in early progeny testing. Anim. Prod. 21, 93-95.

Stickland, N.C., Widdowson, Elsie M. and Goldspink, G. (1975). Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscle of pigs. Brit. J. Nutr. 34, 421-428.

Stickland, N.C. (1975). A detailed analysis of the effects of various fixatives on animal tissue with particular reference to muscle tissue. Stain Tech. 50, 255-264.

Stickland, N.C. and Goldspink, G. (1978). Number of fibres in the skeletal muscle of miniature pigs. J. Agric. Sci. 91, 255-256.

Stickland, N.C. (1978). Muscle weights and succinic dehydrogenase distribution in the hind limb musculature of two rodents (*Thryonomys gegorianus* and *pedetes capensis*) with different locomotory habits. Acta anat. 102, 203-208.

Stickland, N.C. (1979). Comparative aspects of muscle fibre size and succinic dehydrogenase distribution in longissimus dorsi muscle of several species of East African mammals. Acta Anat. 105, (4), 381-385.

Stickland, N.C. (1983). The arrangement of muscle fibres and tendons in tow muscles sused for growth studies. J. Anat. 135, 175-179.

Handel, S.E. and Stickland, N.C. (1986). 'Giant' muscle fibres in skeletal muscle of normal pigs. J. Comp. Path. 96, 447-457.

Stickland, N.C. and Handel, S.E. (1986). The numbers and types of muscle fibres in large and small breeds of pigs. J. Anat. 147, 181-189.

Handel, S.E. and Stickland, N.C. (1987). Muscle cellularity and birthweight. Anim. Prod. 44, 311-317.

Stickland, N.C. (1987). Relationship between motoneuron number and myofibre number. Acta Anat. 128, 214-216.

Handel, S.E. and Stickland, N.C. (1987). The growth and differentiation of porcine skeletal muscle fibre types and the influence of birthweight. J. Anat. 152, 107-119.

Handel, S.E. and Stickland, N.C. (1987). The effects of low birthweight on the ultrastructural development of two myofibre types in the pig. J. Anat. 150, 129-143.

Handel, S.E. and Stickland, N.C. (1988). Catch-up growth in pigs: a relationship with muscle cellularity. Anim. Prod. 47, 291-295.

Zerouala, A.C. and Stickland, N.C. (1991) Cattle at risk from dark cutting beef have a higher proportion of oxidative muscle fibres. Meat Sci. 29, 263-270.

Dwyer, C.M. and Stickland, N.C. (1991). Sources of variation in myofibre number within and between litters of pigs. Anim. Prod. 52, 527-533.

Brown, S.C. and Stickland, N.C. (1993). Satellite cell content in muscles and large and small mice. J. Anat. 183, 91-96.

Dwyer, C.M., Fletcher, J.M. and Stickland, N.C. (1993). Muscle cellularity and postnatal growth in the pig. J. Anim. Sci. 71, 3339-43.

Stickland, N.C., Batt, R.A.L., Crook, A.R. and Sutton, C.M. (1994). Inability of muscles in the obese mouse (genotype, ob/ob) to respond to changes in body weight and activity. J. Anat. 184, 527-533.

Brown, S.C. and Stickland, N.C., (1994). Muscle at birth in mice selected for large and small body size. J. Anat. 184, 371-380.

Stickland, N.C. and O'Shaughnessy, P.J. (1994). The influence of male-specific genes on female muscle fiber types: studies on the sex-reversed (Sxr) mouse. J. Exp. Zool. 269, 378-382.

Stickland, N.C., Crook, A.R. and Sutton, C.M. (1994). The effects of pituitary dwarfism in the mouse on fast and slow skeletal muscles. Acta anat 151, 245-249.

b. ***Published abstracts***

Handel, S.E. and Stickland, N.C. (1984). Muscle cellularity and its relationship and birthweight and growth. J. Anat. 139, 726. (Proceedings of Meeting July 1984, Cambridge).

Handel, S.E. and Stickland, N.C. (1985). The ultrastructural development of two myofibre types (A.271). XII International Anatomical Congress, London, August 1985.

Stickland, N.C. and Handel, S.E. (1985). Muscle cellularity in large and small breeds of pigs (A.678). XII International Anatomical Congress, London, August 1985.

Stickland, N.C., Mescall, Patricia E. and Crook, A.R. (1987). Skeletal muscle characteristics of Snell dwarf mice. J. Anat. 152, 229-230. (Proceedings of Meeting January 1987, Oxford).

Dwyer, C.M. and Stickland, N.C. (1989). The determinants of inter- and intra-litter variation in myofibre number in the pit. J. Anat. 167, 264. (Proceedings of Meeting July 1989, Liverpool).

Stickland, N.C. and O'Shaughnessy, P.J. (1990). The effects of genetic sex and androgens on skeletal muscle tissue. J. Anat. 170, 234-235. (Proceedings of Meeting December 1989, London).

Stickland, N.C., Sutton, Catherine M. and Crook, A.R. (1990). Preparation techniques for automatic counting of skeletal muscle fibres. J Anat. 173, 243-244. (Proceedings of Joint Meeting July 1990, Maastricht, Holland).

Stickland, N.C., Crook, A.R. and Sutton, C.M. (1991). Divergent specialisations of skeletal muscle fibres in cats and dogs. Anatomia Histologia Embryologia 20, 278. (Presented at EAVA XVIII Congress, August 1990, Leipzig).

Rawlinson, S.C.F., Usher, M.L., Dwyer, C.M. and Stickland, N.C. (1991). Localisation of IGF-1 and IGF-2 in a range of vertebrate skeletal muscle tissue. J. Anat. 176, 267. (Proceedings of Meeting December 1990, London).

Stickland, N.C., Batt, R.A.L., Crook, A.R. and Sutton, C.M. (1991). Skeletal muscle in the genetically obese mouse (genotype, ob/ob. J. Anat. 179, 217. (Proceedings of Meeting July 1991, Bristol).

Dwyer, C.M. and Stickland, N.C. (1993). Postnatal growth in the pig and its relationship to muscle fibre number. Anatomia Histologia Embryologia. (Presented at EAVA XIX Congress, August 1992, Ghent).

3. Fish and avian muscle development, growth and adaptation

a. *Original articles*

Stickland, N.C. (1975). Relationship between size of muscle fibres and body dimensions in a number of teleosts. Experientia 31, 1279-1280.

Stickland, N.C. (1977). Succinic dehydrogenase distribution in the pectoralis muscle of several East African Birds. Acta Zool. 58, 41-44.

Stickland, N.C. (1983). Growth and development of muscle in the rainbow trout (*Salmo gairdneri*) J. Anat. 137, 323-333.

Stickland, N.C., White, R.N., Mescall, P.E., Crook, A.R. and Thorpe, J.E. (1988). The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L). Anat. Embryol. 178, 253-257.

Usher, M.L., Stickland, N.C. and Thorpe, J.E. (1994). Muscle development in Atlantic salmon (*Salmo salar* L.) embryos and the effect of temperature on its cellularity. J. Fish Biol. 44, 953-964.

Matschak, T.W. and Stickland, N.C. (1995). The growth of Atlantic salmon *Salmo salar* L.) myosatellite cells in culture at two different temperatures. Experientia 51, 260-266.

Nathanailides, C., Lopez-Albors, O. and Stickland, N.C. (1995). Influence of pre-hatch temperature on the development of muscle cellularity in post-hatch Atlantic salmon (*Salmo salar* L.). Can. J. Fish. Aquat. Sci. 52, 675-680.

Nathanailides, C., Lopez-Albors, O. and Stickland, N.C. (1995). Temperature- and developmentally-induced variation in the histochemical profile of myofibrillar ATPase activity in carp (*Cyprinus carpio* L.). J. Fish Biol. 47, 631-640.

Matschak, T.W., Stickland, N.C., Crook, A.R. and Hopcroft, T. (1995). Is physiological hypoxia the driving force behind temperature effects on muscle development in embryonic Atlantic salmon (*Salmo salar* L.)? Differentiation 59, 71-77.

Matschak, T.W. & Stickland, N.C. (1995). The influence of temperature on mRNA levels for muscle contractile protein and a proto-oncogene associated with cell division in Atlantic salmon (*Salmo salar* L.). Can. J. Fish. Aquat. Sci. 53, 408-413.

Nathanailides, C. & Stickland, N.C. (1996). Activity of cytochrome c oxidase and lactic dehydrogenase in muscle tissue of slow growing (lower modal group) and fast growing (upper modal group) Atlantic salmon. J. Fish Biol. 48, 549-551.

Nathanailides, C., Lopez-Albors, O., Abellan, E., Vazquez, J.M., Tyler, D.D., Rowlerson, A. and Stickland, N.C. (1996). Muscle cellularity in relation to somatic growth in the European sea bass *Dicentrarchus labrax*, L. Aquaculture Research 27, 101-105.

b. *Published abstracts*

Brooks, A.R.B. and Stickland, N.C. (1986). A comparison of light and electron microscopic studies on muscle growth in Atlantic salmon (*Salmo salar*, L.). J. Anat. 146, 252. (Proceedings of Meeting January 1986, Southampton).

Usher, M.L. and Stickland, N.C. (1989). The role of temperature in muscle development in Atlantic salmon (*Salmo salar* L.) embryos. J. Anat. 167, 254. (Proceedings of Meeting July 1989, Liverpool).

Matschak, T.W., Ennion, S., Goldspink, G. and Stickland, N.C. (1993). Effect of temperature on muscle protein mRNA levels in embryonic salmon (*Salmo salar* L.). J. Muscle Res. Cell Motility 14, 249. (Presented at XXI European Muscle Congress, Sept. 1992. Bielefeld, Germany).

Nathanailides, C., Lopez-Albors, O. and Stickland, N.C. (1993). The growth response of the lateral musculature of carp (*Cyprinus carpio* L.) to temperature. J. Anat. 183, 184-185. (Proceedings of Meeting January 1993, London).

Matschak, T.W., Stickland, N.C. and Thorpe, J.E. (1994). Does the chorion have an effect on the development of muscle cellularity in prehatch Atlantic salmon (*Salmo salar* L.)? J. Muscle Res. Cell Motility 15, 189-190. (Presented at XXII European Muscle Congress, Oct. 1993, Gwatt, Switzerland).

Nathanailides, C., Lopez, O. and Stickland, N.C. (1994). Effect of prehatch temperature on the posthatch growth of muscle in the Atlantic salmon. J. Anat. 185, 705. (Proceedings of Meeting April 1994, Utrecht, Netherlands).

Matschak, T.W., Stickland, N.C., Mason, P.S. and Crook, A.R. (1997). Muscle development in salmonids at different temperatures and oxygen levels. Anat. Histol. Embryol. (in press). (Proceedings of XXI EAVA Congress July 1996, Lugo, Spain).

Matschak, T.W. and Stickland, N.C. (1997). Differences in muscle differentiation of Atlantic salmon (*Salmo salar* L.) embryos in response to temperature and oxygen levels at a late developmental stage. J. Anat. (in press). (Proceedings of Meeting December 1996, London).

1. Prenatal mammalian development including myogenesis and placentation

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Department of Veterinary Anatomy and Histology,
University of Nairobi

A Quantitative Evaluation of Placentome Development in Zebu Cattle (Bos indicus)

By

N. C. Stickland and M. D. Purton

With one figure and one table

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Introduction

It has been established that the bovine cotyledonary placenta develops from the fusion of maternal caruncles, present in the uterus of the non-pregnant animal, with fetal cotyledons (localized tufts of chorionic villi) (Amoroso, 1952). These specialized mucosal surfaces form the typical placentomes of the pregnant animal.

The complete dependancy of the developing bovine embryo upon the placenta, and more particularly upon the placentomes, for all its metabolic requirements is not established until around the fiftieth day of gestation (Smidt, 1951). The way in which the developing embryo receives its nutritional requirements prior to this stage has been fully investigated (Green, 1946; Melton, 1951) while extensive morphological studies have also provided much qualitative information on placental/placentome development after this stage (Boyd et al., 1944; Hafez/Rajakoski, 1964). However, quantitative studies of placentome development from the fiftieth day onwards appear to be lacking in the literature.

The purpose of the present investigation was to study in detail the quantitative changes that occur during placentome development in Zebu cattle.

Material and Methods

Fetuses "in utero" were collected from 35 Zebu cows *(Bos indicus)* collected from the same environment and slaughtered at a local abattoir at the same time of year. Each specimen was stored in 10 % Formalin for transport purposes. The cows were in various stages of pregnancy, so that fetuses ranged in age from 75 to 245 days of gestation (as calculated from the data of Evans/Sack, 1973).

The following procedure was used for studying each fetus and placenta. Both horns of the uterus were opened and the fetus removed so that the crown-rump length could be recorded. The number of placentomes in both the gravid and the non-gravid horn was then noted, and the major and minor diameter of every placentome measured using calipers.

Calculations were based upon the assumption of an elliptical circumference for each placentome (an assumption based upon present observations). The cross-sectional area of each placentome could therefore be calculated according to the equation for the area of an ellipse, where Area $= \frac{\pi AB}{4}$ (A and B being the major and minor diameter respectively). It should be noted that this cross-sectional area was the largest sectional area of the placentome taken parallel to the uterus wall, and is referred to as placentome area in the rest of the text. In thirteen placentae the depth of the placentomes was measured in the centre (after cutting through the middle, perpendicular to the uterus wall) as well as the volume of the placentomes (by displacement in a graduated cylinder).

Correlation coefficients and lines of regression were calculated for crown-rump length against number of placentomes, mean placentome area (average area of a single placentome) and total placentome area (total area of all placentomes) for each animal. All calculations were carried out separately for gravid and for non-gravid horns, and also for both horns combined. Statistical calculations were based on methods given by SNEDECOR/COCHRAN (1967).

Results

Table 1 shows the correlation coefficients and formulae for the lines of regression for crown-rump length plotted against the various parameters investigated. A strong correlation between crown-rump length and both mean placentome area and total placentome area is apparent; no such correlation exists between crown-rump length and placentome number, suggesting no change in number of placentomes with age. The mean number of placentomes was found to be 69.0 ($\pm$ 2.6), and of this total 41.0 ($\pm$ 1.5) were located in the gravid horn and 28.1 ($\pm$ 1.5) in the non-gravid horn. The difference in numbers between gravid and non-gravid horns was highly significant ($P < 0.001$). The equations for the regression lines presented in Fig. 1 are is given in Table 1.

The lines in Fig. 1 (b) are not significantly different from the horizontal. However, in Figs. 1 (a) and (c), the slopes of the lines for the gravid horns are significantly steeper ($P < 0.005$) than the slopes of the lines for the non-gravid horns. Although individual points are not represented in the figure, the standard error of an estimation of Y based upon X (crown-rump length) is given in Table I.

Table 1

Correlation Coefficients and Formulae of regression lines

		n	r	P	Regression line	$S_{y.x}$
Crown-rump length (X)						
v. Mean Placentome Area (Y)	Gravid Horn	35	0.947	<0.001	Y = 0.49.X - 4.04	± 0.49
	Non-gravid Horn	35	0.889	<0.001	Y = 0.28.X - 3.38	± 0.43
	Both Horns	35	0.938	<0.001	Y = 0.40.X - 3.58	± 0.43
v. Number of Placentomes (Y)	Gravid Horn	35	0.120	N. S.	Y = 0.06.X + 38.96	± 1.53
	Non-gravid Horn	35	0.186	N. S.	Y = 0.10.X + 24.95	± 1.50
	Both Horns	35	0.177	N. S.	Y = 0.16.X + 63.91	± 2.61
v. Total Placentome Area (Y)	Gravid Horn	35	0.918	<0.001	Y = 21.31.X - 208.80	± 27.4
	Non-gravid Horn	35	0.905	<0.001	Y = 9.17.X - 136.11	± 12.9
	Both Horns	35	0.933	<0.001	Y = 30.48.X - 344.84	± 34.9
Mean Placentome Area (X)						
v. Mean Placentome Volume (Y)	Both Horns	13	0.957	<0.001	Y = 1.63.X - 4.21	± 0.64
v. Mean Placentome Depth (Y)	Both Horns	13	0.905	<0.001	Y = 0.11.X + 0.23	± 0.07

N. S. = Not Significant
r = Correlation coefficient
P = Significance of r
$S_{y.x}$ = Standard Error of Y estimated from X

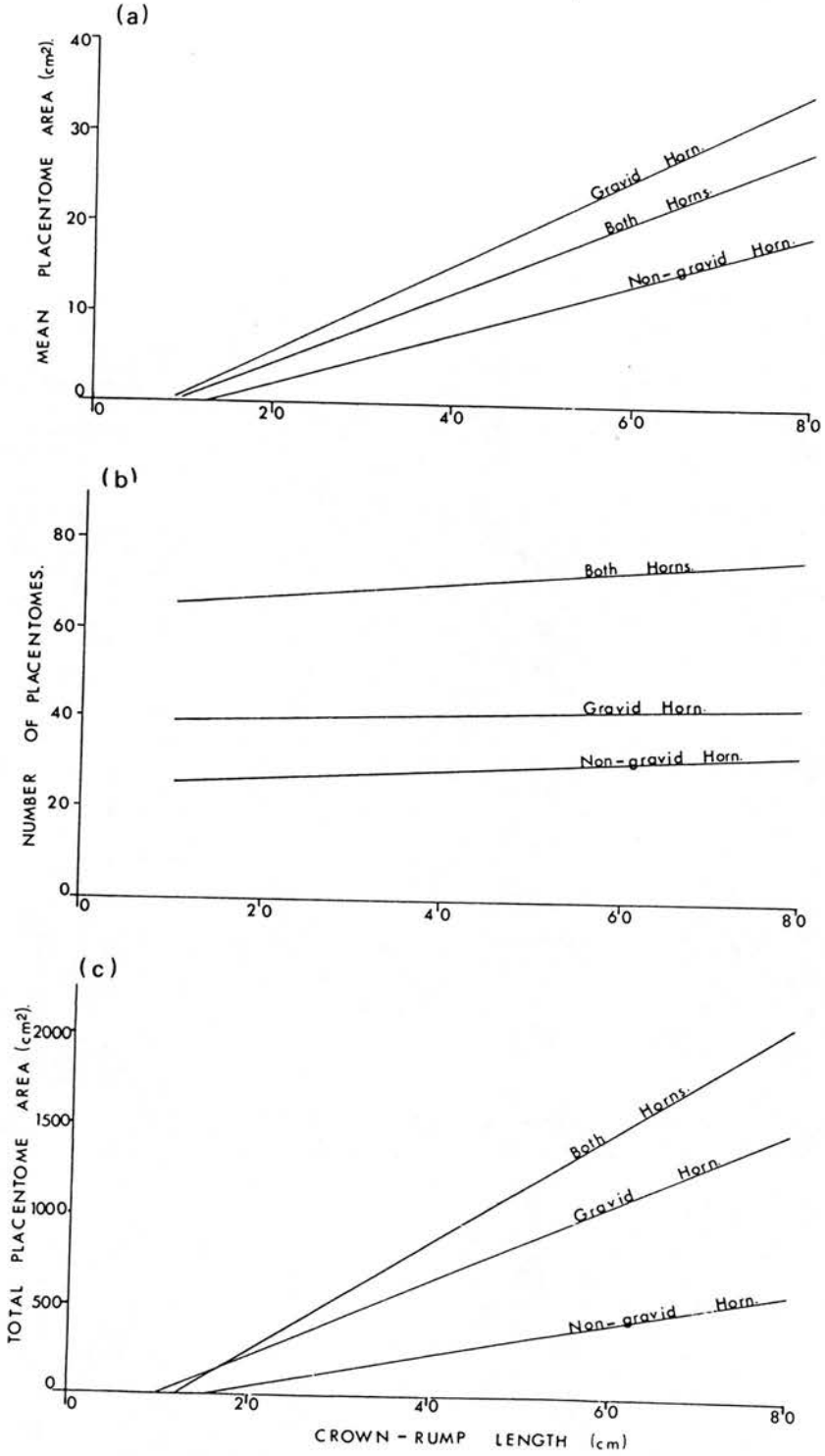

Fig. 1. Relationship between Crown-Rump length of fetus and (a) Mean Placentome Area, (b) Number of Placentomes and (c) Total Placentome Area. (See text for definition of Area)

It was also found, using the sample of thirteen placentae, that the area of the placentomes was very closely related to the depth and to the volume of the placentomes (Table 1).

It should be noted here that all measurements were made on fixed material so that there was inevitably some distortion. STICKLAND (1975) found that 10 % Formalin (with no further processing) caused the volume of animal tissue to increase by 6 % after 24 hours in fixative. This will mean an even smaller increase in cross-sectional area (about 3.3 %) and so can be considered negligible in this investigation.

Discussion

The present study has shown that the placentomes increase in mean area as the crown-rump length of the fetus increases from 10 cm. to 80 cm. As crown-rump length is linearly related to duration of the gestation period (EVANS/SACK, 1973), it would seem that, from the present data, placentome area increases continuously from about 75 days gestation to near birth. Placentome numbers, however, remained constant throughout the present period of study. Increase in total placentome area can be explained therefore by an increase in size of placentomes alone. Such an observation appears to contradict HAMMOND (1927) who has stated that, in the cow, placentome development is completed by the third or fourth month; however, his meaning of development is unclear.

Such increase in total placentome area shown in the present study is similar to that found by KAYANJA (1973) in the impala. His equation for the regression of total placentome area on crown-rump length ($Y = 18.5\ X - 127$), and the corresponding correlation ($r = 0.96$), were of the same order as those given here in Table 1. This situation is markedly different from that in the sheep, where CLOETE (1939) showed that the diameter of the placentomes increased up to the third month, then decreased. It also appears that in the sheep the placentomes in the gravid horn are only about 6 % larger in diameter than those in the non-gravid horn (this is approximately equivalent to a 12 % difference in area). Present observations have shown that the mean placentome area in the gravid horn is almost twice as great as that in the non-gravid horn at all stages of gestation, while total placentome area in the gravid horn is some three times greater than in the non-gravid horn at the 20 cm crown-rump length stage, and some two and a half times greater at the 80 cm. stage. The close relationship found between placentome area and depth, and between placentome area and volume suggests that the changes shown here for placentome area are probably meaningful in describing other dimensional changes in placentomes.

The total number of placentomes was found to average 69 in the present study. This is significantly lower than figures of 70 to 142 (varous authorities quoted by AMOROSO, 1952) for the cow. However, the present low numbers could be related to the relatively poor nutritional grazing available to local cattle, a factor which has been shown to affect placentome number in the ewe (EVERITT, 1968). On average, there were found to be about 50 % more placentomes in the gravid than non-gravid horns. This is in agreement with similar results for the cow (RORIK, 1907), impala (KAYANJA, 1973) and ewe (EVERITT, 1968).

Acknowledgements

We would like to thank Mr. S. Paye for technical assistance.

Summary

Quantitative changes occurring during placentome development were studied in detail in fetuses of local Zebu cattle ranging in age from about 75 to 245 days of gestation. Total placentome cross-sectional area increased continuously throughout gestation, although placentome number remained constant; this was brought about by an increase in mean placentome cross-sectional area alone. Mean placentome cross-sectional area in the gravid horn was almost twice as great as that in the non-gravid horn at all stages of gestation. Changes in placentome cross-sectional area were closely related to changes in placentome depth and volume.

Zusammenfassung

Quantitative Erhebungen zur Plazentomentwicklung beim Zebu (Bos indicus)

Die quantitativen Veränderungen der Plazentomentwicklung wurden an Zebu-Föten im Trächtigkeitsalter von 75—245 Tagen studiert. Bei gleichbleibender Anzahl der Plazentome nimmt deren Gesamtquerschnittsfläche während der Trächtigkeit ständig zu, und zwar als Folge der Zunahme der mittleren Flächengröße der einzelnen Plazentome, die durchweg im trächtigen Horn fast doppelt so groß war wie im nichtträchtigen. Diese Änderungen hängen mit Änderungen der Höhe und des Umfanges der Plazentome eng zusammen.

Résumé

Evaluation quantitative du développement des placentomes chez le zébu (Bos indicus)

Les changements quantitatifs qui se produisent pendant le développement des placentomes ont été étudiés chez des fœtus de Zébu de race locale âgés de 75 à 245 jours de gestation. La surface totale des placentomes en coupe transversale augmente de façon continue pendant toute la gestation, bien que le nombre de placentomes reste constant. Ceci est réalisé par la seule augmentation de la surface moyenne de section des placentomes. Cette moyenne s'est avérée presque deux fois plus grande dans la corne gravide que dans la corne non gravide à tous les stades de la gestation. Les changements ont été liés à ceux du volume des placentomes.

Resumen

Una evaluación cuantitativa del desarrollo de los placentomas del cebú (Bos indicus)

Los cambios cuantitativos durante el desarrollo de los placentomas se estudiaron en fetos del cebú de aproximadamente 75 a 245 días de edad. Durante la gestación, la superficie total de cortes transversos de los placentomas aumenta constantemente, manteniéndose su número igual; esto se debe sólo al aumento de superficie cuyo promedio es casi el doble en el cuerno grávido en comparación con el otro. Estos cambios están intimamente relacionados con los cambios de los placentomas en altura y volumen.

References

Amoroso, E. C., 1952: Placentation. In: Marshall's Physiology of Reproduction. A. S. Parkes (Ed.), Chapter 15. Volume II. 3rd Edition. Longmans, Green, London.

Boyd, J. D., W. J. Hamilton and J. Hammond, 1944: Transuterine ("internal") migration of the ovum in sheep and other mammals. J. Anat. *78*, 5.

CLOETE, J. H. L., 1939: Prenatal growth in the Merino sheep. Onderstepoort. J. Vet. Sci. *13*, 417.

EVANS, H. E., and W. O. SACK, 1973: Prenatal development of domestic and laboratory mammals: Growth curves, external features and selected references. Anat., Histol., Embryol. *2*, 11.

EVERITT, G. C., 1968: Prenatal development of uniparous animals with particular reference to the influence of maternal nutrition in sheep. In: Growth and Development of Mammals. G. A. LODGE and G. E. LAMMING (Ed.), Butterworths, London.

GREEN, W. W., 1946: Comparative growth of the sheep and bovine animal during prenatal life. Am. J. Vet. Res. *7*, 395.

HAFEZ, E. S. E., and E. RAJAKOSKI, 1964: Placental and foetal development during multiple bovine pregnancy. Anat. Rec. *150*, 303.

HAMMOND, J., 1927: The Physiology of Reproduction in the Cow. Cambridge University Press, London and New York.

KAYANJA, F. I. B., 1972: Reproduction in Antelopes. East African Monographs in Biology. Volume I. East African Literature Bureau, Nairobi.

MELTON, A. A., R. O. BERRY and O. D. BUTLER, 1951: The interval between time of ovulation and attachment of the bovine embryo. J. Anim. Sci. *10*, 992.

RORIK, H. H., 1907: Berechnung der Oberfläche der Uteruskarunkeln (semi-placenta materna) beim Rind. Arch. wiss. prakt. Tierheilk. *33*, 421.

SMIDT, G. A., 1951: Periods of embryonic development in cattle. Dokl. Akad. Nauk S. S. S. R. *80*, 137.

SNEDECOR, G. W., and W. G. COCHRAN, 1967: Statistical Methods. 6th Edition. The Iowa State University Press, Ames. Iowa.

STICKLAND, N. C., 1975: A detailed analysis of the effects of various fixatives on animal tissue with particular reference to muscle tissue. Stain Tech. *50*, 255.

Authors' present addresses: Dr. N. C. STICKLAND, Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH, U. K.

Dr. M. D. PURTON, Department of Veterinary Anatomy, University of Glasgow, Bearsden, Glasgow, U. K.

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Department of Veterinary Anatomy, University of Nairobi
and Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh

A Quantitative Study of Muscle Development in the Bovine Foetus (Bos indicus)

By

N. C. STICKLAND

With 9 figures and one table

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Abstract

The investigation was carried out in order to quantify the changes in various cell parameters which take place in the normal prenatal development of bovine muscle. Sections of muscles, taken from 36 Zebu foetuses *(Bos indicus)* of about 75 to 245 days gestation, were examined microscopically. Results are given on the numbers and sizes of myotubes and myofibres seen in section at various stages of development. The relative contribution of increases in cell size and cell number to overall muscle size increase are discussed.

Introduction

It is now well established by many authors (MACCALLUM, 1898, in the human; STAUN, 1963, and STICKLAND/GOLDSPINK, 1973, in the pig; ROWE/GOLDSPINK, 1969, in the mouse; ELIOT et al., 1943, in the rat) that the total number of fibres in a given muscle is fixed at or near birth, the exact time probably depending on the maturity of the animal at the time of birth. This fixed number of muscle fibres is not affected by even very severe levels of undernutrition during postnatal growth (STICKLAND et al., 1975), whereas the size of the muscle fibres can be affected by many factors such as age (HAMMOND/APPLETON, 1932; JOUBERT, 1956; STICKLAND/GOLDSPINK, 1973), exercise (GOLDSPINK, 1964) and level of nutrition (JOUBERT, 1956; STICKLAND et al., 1975).

It is evident that the total number of fibres in a given muscle must be increasing during prenatal development until the genetically determined adult number is reached. The processes involved in early myogenesis have been studied quite extensively by using *in vitro* techniques (see HOLTZER/BISCHOFF,

1970). Histochemical techniques have also been used to study aspects of prenatal muscle fibre type differentiation in the lamb (ASHMORE et al., 1972), pig (ASHMORE et al., 1973; SWATLAND/CASSENS, 1973) and bovine foetus (OMMER, 1971).

Although these two lines of investigation have produced interesting results, there appears to have been no attempt to quantify the changes in size and number of the cellular parameters which occur during prenatal muscle development in agricultural animals. This is with the possible exceptions of JOUBERT (1955, 1956) and SWATLAND/CASSENS (1973 a) working on foetal sheep and THURLEY (1972) on foetal pigs. As far as bovine foetal muscle is concerned, detailed information on changes in size of cellular components appears to be lacking. A statistically significant study of cell numbers in foetal muscle appears to be lacking in all agricultural animals which is surprising when it is realised how important total muscle fibre number is in relation to the meat content of an animal (STAUN, 1968; STICKLAND/GOLDSPINK, 1975).

A detailed time-scale analysis of the quantitative changes taking place in normal prenatal muscle development would be of value to the pathologist studying developmental myopathies and to the meat scientist or muscle histologist interested in muscle fibre size and number. The opportunity was therefore taken of investigating various cellular parameters of muscles taken from Zebu foetuses of varying gestation periods. The emphasis of the investigation was on quantifying the changes that take place.

Material and Methods

Foetuses were collected from 36 Zebu cows *(Bos indicus)* slaughtered at Athi River Abattoir, near Nairobi, Kenya. The animals had been reared in the same environment and were all slaughtered at the same time of year. The cows were in various stages of pregnancy, so that foetuses ranged from 9 · 4 to 80 cm. in crown-rump length (CRL, which is about 75 to 245 days gestation according to the data of EVANS/SACK, 1973). All foetuses were placed in 10 % formalin immediately after slaughter for storage and transport purposes.

In the laboratory the procedure was the same for each foetus. The CRL was first measured and then *m. peroneus longus* removed entire from the hind limb. The muscle was cleared of connective tissue, weighed and then processed (according to STICKLAND, 1975, for 10 % formalin fixation) in order to obtain 10 μm paraffin sections made transversely across the widest part of the muscle and stained in haematoxylin and eosin. By means of a Leitz projection microscope, the area of this cross-section was outlined on paper and the area estimated by the paper-weighing method. From the data of STICKLAND (1975), the area thus obtained from this 10 % formalin-fixed muscle would be only about 48 % of the cross-sectional area of the fresh muscle. However, as the results were used comparatively, no adjustments were made. The myotube and myofibre diameter measurements were also not adjusted for shrinkage for similar reasons.

A small piece of *m. longissimus dorsi* taken from the thoraco-lumbar junction was also removed. This tissue sample was washed, post-fixed in 1 % OsO_4, washed, and then processed through a series of graded alcohols and propylene oxide before embedding in araldite. 1 μm transverse sections were cut on an ultramicrotome and stained with 1 % toluidine blue. All sections were photographed using a Leitz microscope with camera attachment (automatic exposure). The area covered in each photograph was estimated to be 0 · 0109 sq. mm. In each of the enlarged prints the total number of myotubes (with centres devoid of myofibrils), nuclei and myofibres (uniform spread of myofibrils) was counted. For 12 foetuses the diameters of 100 cellular elements was measured in the section by means of an eye-piece graticule in the microscope. The proportion of myotube and myofibre diameters measured depended on the ratio of each estimated from the photograph counts mentioned above.

Correlation coefficients and lines of regression of various parameters on CRL were calculated by the methods given by SNEDECOR/COCHRAN (1967).

Results

Fig. 1 shows the increase in weight and Fig. 2 the increase in cross-sectional area of *m. peroneus longus* as the CRL of the foetus increases.

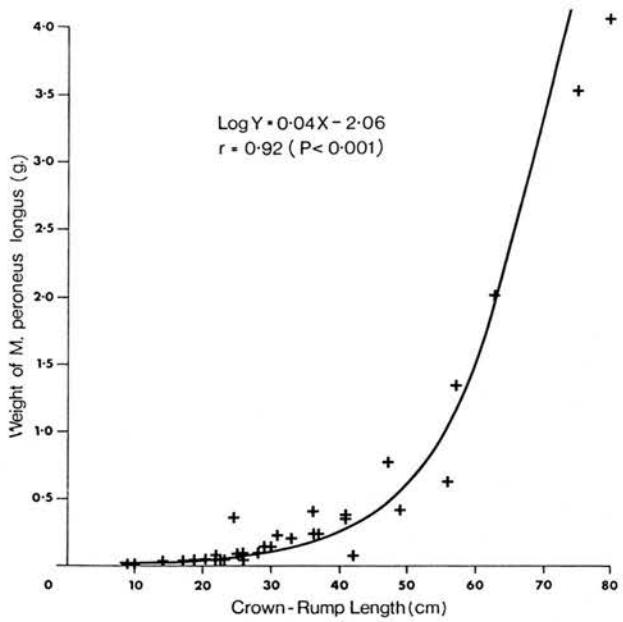


Fig. 1. Relationship between CRL of foetus and weight of *m. peroneus longus*

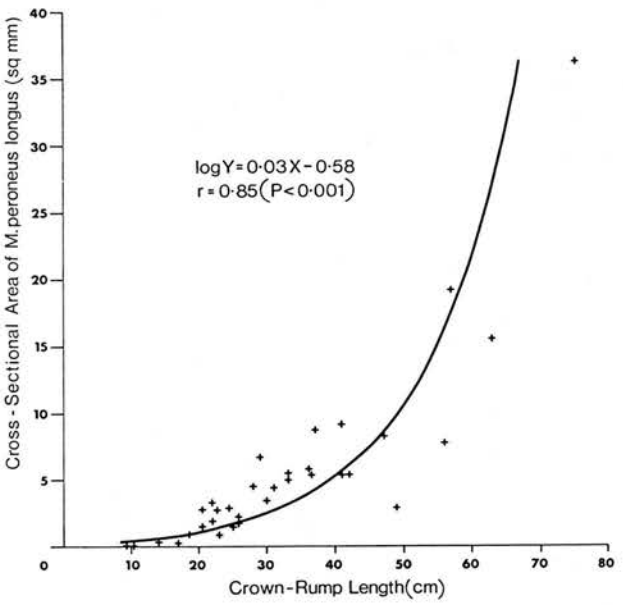
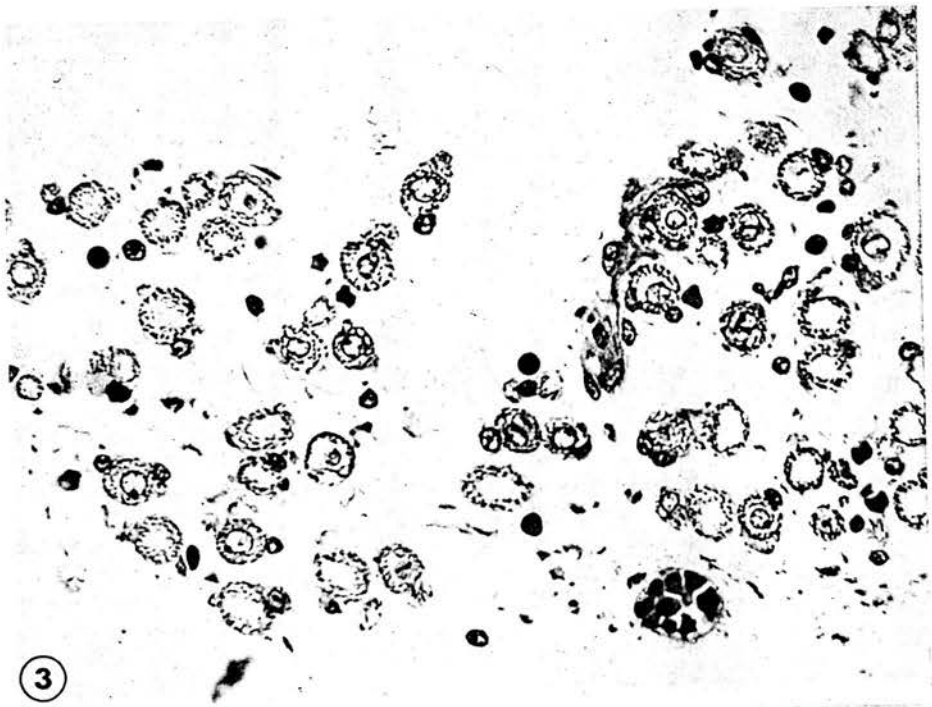


Fig. 2. Relationship between CRL of foetus and cross-sectional area of *m. peroneus longus*

Both regression lines are logarithmic with significant correlations. In both figures "X" is the CRL.

Photomicrographs of four stages of muscle development are shown in Figs. 3 to 6. The approximate gestation times given are calculated from the CRL according to EVANS/SACK (1973). Large myotubes can be clearly seen in Fig. 3 and smaller myotubes as well as many myofibres in Fig. 4. At this stage (Fig. 4), the perimysium appears to be forming the cellular elements into fasciculi, although the myotubes and myofibres in the fasciculi do not appear to be arranged in any regular manner. The arrangement into fasciculi is continued in Fig. 5 together with an increase in myofibre size and loss of myotubes. Fig. 6 shows a greater increase in myofibre size as well as a pronounced decrease in intercellular space. Fig. 7 shows exactly how the diameters of these myotubes and myofibres change as the foetus grows. The myotubes decrease in diameter whilst they are present with the myofibre diameter also showing a concomitant slight decrease, but then clearly increasing in diameter as soon as the myotubes disappear. There is also also a wider diameter distribution of myofibres as the foetus grows.



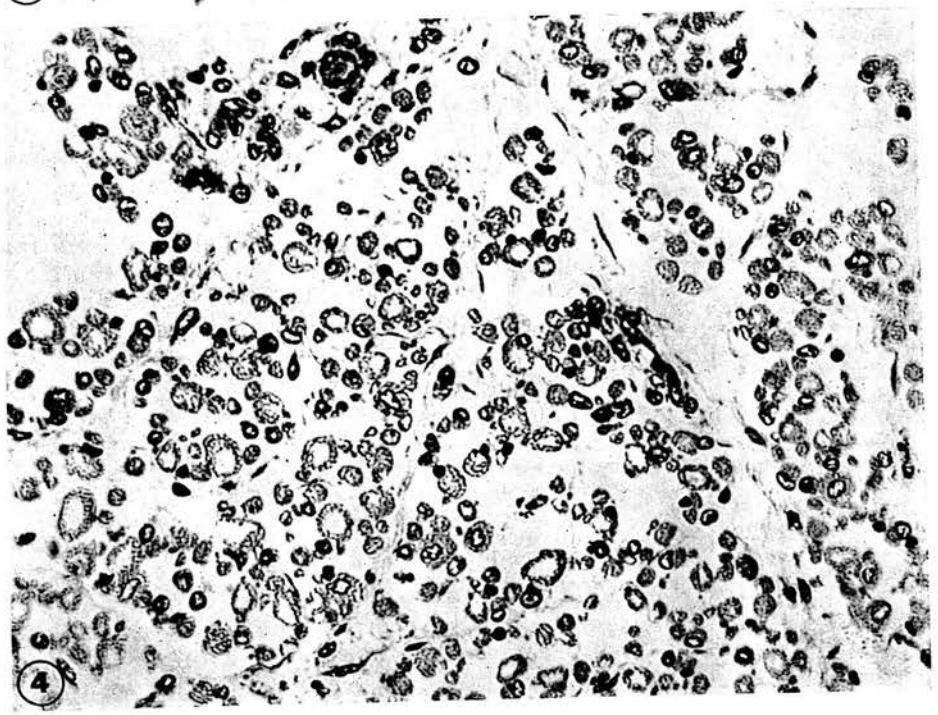
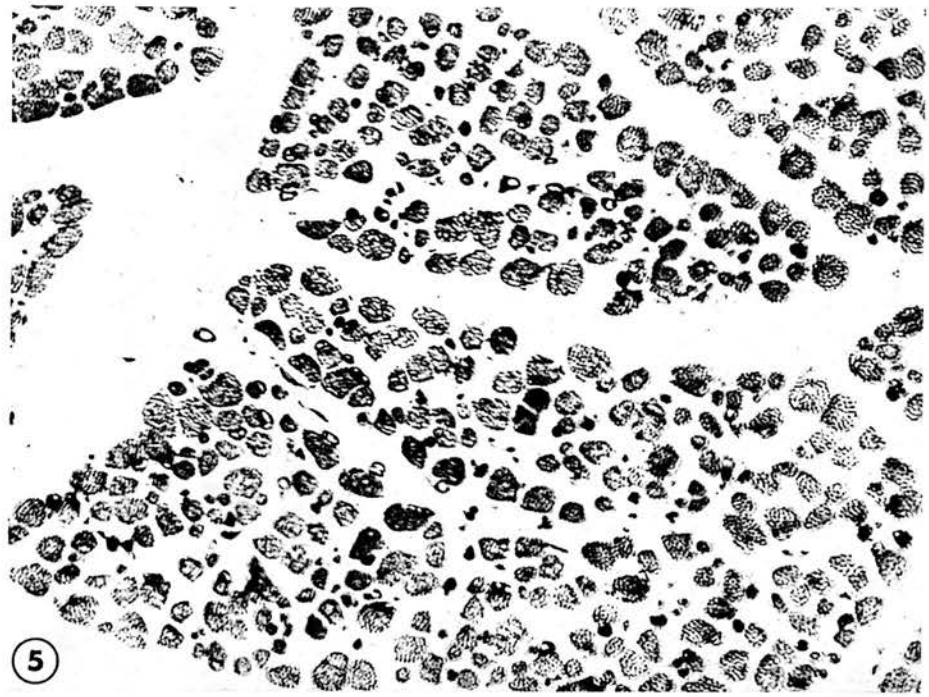


Fig. 3. T. S. *m. longissimus dorsi*. CRL 9 · 4 cm. (approx. 75 days gestation). × 600
Fig. 4. T. S. *m. longissimus dorsi*. CRL 25 · 8 cm. (approx. 125 days gestation). × 600



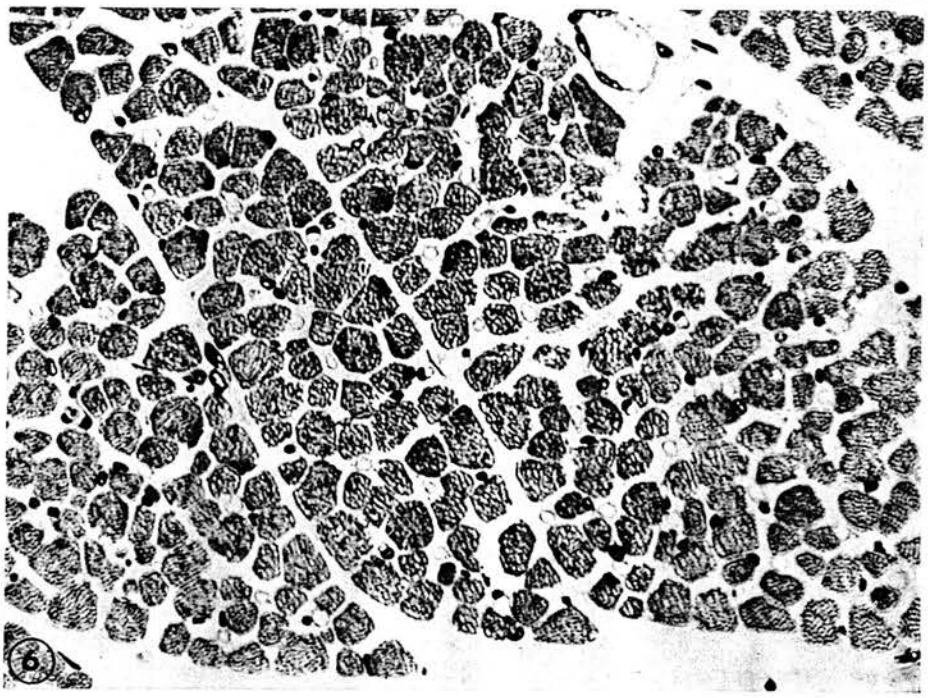


Fig. 5. T. S. *m. longissimus dorsi.* CRL 47 cm. (approx. 177 days gestation). × 600
Fig. 6. T. S. *m. longissimus dorsi.* CRL 75 cm. (approx. 235 days gestation). × 600

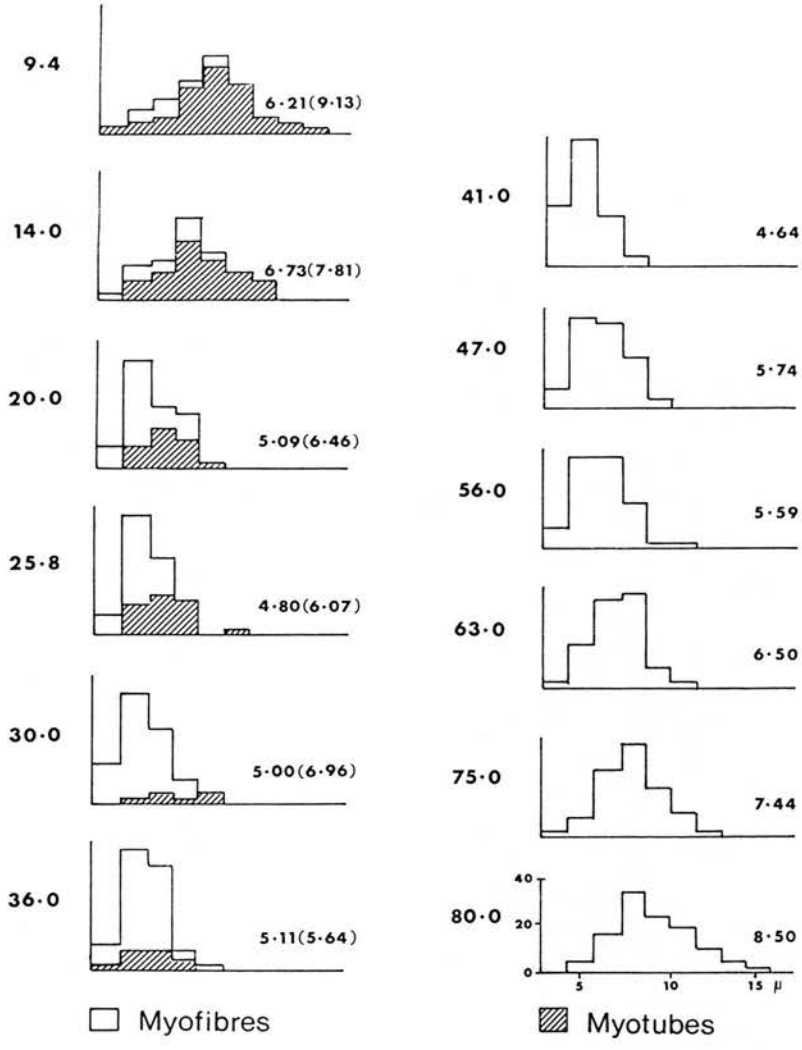


Fig. 7. Histograms showing the change in myofibre and myotube diameter distribution in *m. longissimus dorsi* with increase in CRL of the foetus. The number to the left of each histogram is the CRL in cm. The mean myofibre diameter is given on each histogram in μm, with the mean myotube diameter in brackets

Fig. 8 shows how the number of myofibres, myotubes and nuclei per unit area (estimated at 0 · 0109 sq. mm. as mentioned above) changes with increase in CRL. The lines shown are all computed lines of regression, the formulae of these lines being shown in Table 1. The dotted lines are extrapolations of the computed lines. Owing to the complex nature of this graph it was necessary to compute the myofibre regression lines in two stages dividing the points at 30 cm. where an obvious change seemed to be occurring. The number of myofibres shows a pronounced increase from about 10 to 30 cm. (line 1) whereas the number of myotubes decreases to zero (line 5) by about 35 cm. Both lines are significant (Table 1). Using the myofibre points from 30 to 80 cm., line 2 is obtained but is not significantly different from

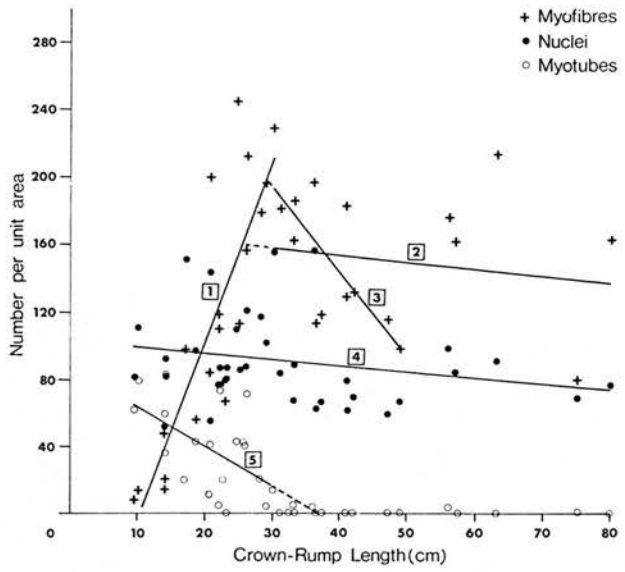


Fig. 8. Relationship between CRL of foetus and density of myofibres, myotubes and nuclei in cross-section of *m. longissimus dorsi*

the horizontal, which may be due to the large variation between the last four points (55 to 80 cm.) so that no definite conclusion can be made on this line. However, if the points between 30 and 50 cm. only are used, then line 3 is obtained which is statistically significant (Table 1). The numbers of nuclei per unit area (line 4) do not change during the time period investigated, the line shown not being significantly differeit from the horizontal (Table 1).

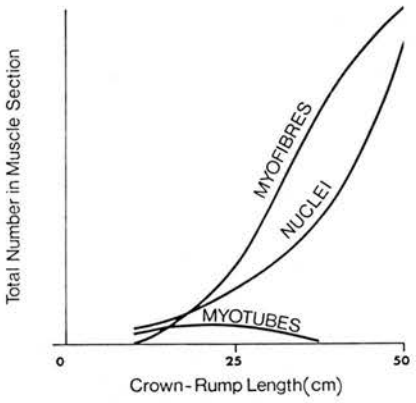


Fig. 9. A theoretical figure suggesting the change in the total number of myofibres, myotubes and nuclei in a muscle cross-section with increase in CRL of the foetus

Table 1
Correlation coefficients and formulae of regression lines shown in Fig. 8

	n	r	P (of r)	Regression line	P (of b)
Crown-Rump length (X)					
[1] v. Number of Myofibres (Y)	20	0·840	<0·001	Y = 10·67. X - 108·4	<0·001
[2] " " " " "	16	-0·161	N. S.	Y = -0·42. X + 171·7	N. S.
[3] " " " " "	13	-0·762	<0·001	Y = -4·97. X + 344·4	<0·001
[4] " " " Nuclei (Y)	36	-0·240	N. S.	Y = -0·38. X + 103·7	N. S.
[5] " " " Myotubes (Y)	20	-0·529	<0·02	Y = -2·31. X + 86·8	<0·01

([1] - [5] = Number of line shown in Fig. 8; b = Regression coefficient; n = Number of animals; P = Significance; r = Correlation coefficient; v. = Versus; N. S. = Not significant.)

Discussion

The growth of the bovine foetus illustrates a typical logarithmic prenatal growth curve (Winters et al., 1942) so that most of the foetal weight increase occurs in the later stages of gestation. The logarithmic curves shown in Figs. 1 and 2 are, therefore, to be expected. Joubert (1956) found a similar relationship between weight and dimensions of various muscles and age of foetal sheep. As crown-rump length is almost linearly related to foetal age (Evans/Sack, 1973), then age could be substituted in this investigation without altering the shape of the curve to any great extent. It should be noted here that, although regression lines of all bovine muscles would be of the same form as those given in Figs. 1 and 2, the actual regression coefficient values would probably vary to some extent between muscles owing to the slightly different prenatal growth rates of different muscle groups (Joubert, 1956).

From this investigation it would seem that the precursors of muscle fibres in the bovine foetus are either large myotubes seen at an early stage (Fig. 3) or smaller cells (with or without a myotube appearance) which develop between these larger myotubes at a later stage (Fig. 4). This biphasic muscle development was put forward by Wohlfart (1937) and has been shown in the pig (Ashmore et al., 1973; Swatland/Cassens, 1973), lamb (Ashmore et al., 1972), rat (Kelley/Zacks, 1969) and chick (Kikuchi, 1971). Using histochemical techniques, it has been shown in the pig (Ashmore et al., 1973) and lamb (Ashmore et al., 1972) that the large myotubes which develop first are probably destined to become slow-twitch fibres of adult muscle and were termed presumptive β-fibres. In contrast, the smaller cells which develop later are probably destined to become fast-twitch fibres and were termed presumptive α-fibres. Photomicrographs in these papers showed how the presumptive β-fibres appeared to be the framework around which the presumptive α-fibres developed. This theory was also propounded by Kelley/Zacks (1969) in rat muscle. Ommer (1971) demonstrated very early differentiation of muscle fibres in the bovine *longissimus dorsi* muscle (using succinic dehydrogenase activity), but the fibre type distribution was not the regular pattern seen in the studies mentioned on pig and lamb. It is also difficult to see such a regular pattern in this present investigation (Fig. 4).

All investigations have shown that the presumptive β-fibres have a myotube appearance (peripheral myofibrils). The presumptive α-fibres, however, were described as small myotubes by Ashmore et al., (1972, 1973) but as small myofibres with a core of myofibrils (termed secondary foetal myofibres with β-fibres being the primary foetal myofibres) by Swatland/Cassens (1973). In this present investigation it was difficult to distinguish small myotubes from small myofibres in the population of small cellular elements, in contrast to the large myotubes which were very obvious. The generally accepted view, however, is that myoblasts fuse to form myotubes which then differentiate into myofibres. It seems most likely, therefore, from all the evidence, that myoblasts surrounding the large primary myotubes fuse to form small secondary myotubes which differentiate relatively quickly into myofibres, whereas the large primary myotubes take longer to attain a myofibre appearance. Hence, the population of small cells shows a transition from myotube to myofibre appearance (Fig. 4). The appearance of the early myotube (Fig. 3) is that of a large cell with peripheral myofibrils and a large central "vesiculated" nucleus. This description of a myotube was first given by MacCallum in 1898 who also described the presence of more solid, darker nuclei at the periphery of these cells in embryonic pig muscle. These darker peripheral nuclei can be seen here and were also

described by THURLEY (1972) and SWATLAND/CASSENS (1973) who suggested that they are the nuclei of mononucleate cells which are possibly destined to become "secondary foetal myofibres". Fig. 4 shows how the connective tissue is starting to arrange the myotubes and myofibres into bundles or fasciculi. As also observed by DANILOVA (1960) in the bovine foetus, this perimysium is apparently organised before the endomysium which can be seen at a later stage (Figs. 5, 6).

The distributions shown in Fig. 7 demonstrate a change from a flat-topped histogram of high mean diameter at 9 · 4 cm. CRL to a narrow distribution with a low mean diameter at 41 · 0 cm., and then reverting to a flat-topped histogram with a high mean diameter at 80 · 0 cm. It must be remembered that the diameters given in Fig. 7 were estimated from 10 % formalin-fixed muscle tissue. These are approximately 69 % of the probable fresh muscle fibre diameters (STICKLAND, 1975). These changes (Fig. 7) are in agreement with the results of THURLEY (1972) for the pig and JOUBERT (1956) for the lamb. Neither of these authors, however, distinguished between myotubes and myofibres in their histograms. It is clear from Fig. 7 that the initial high mean diameter observed by these authors is due to the large diameter of the early myotubes which decrease in size and number until, at about 40 cm., all myotubes have presumably attained the appearance of myofibres. The results given here indicate that there may be a slight initial decrease in myofibre diameter during the myotube stage, although this could be due to inadequate distinction of small myotubes and myofibres (as mentioned above). However, JOUBERT (1956) found that just before half-way through gestation in the lamb the incidence of smallest fibres was greatest, and this can be seen here (Fig. 7) from 30 to 41 cm. It is quite clear from Fig. 7 that the period from 41 · 0 cm. to 80 · 0 cm. shows a marked increase in myofibre diameter, and this is also evident in Figs. 5 and 6. JOUBERT (1956) also stressed that the largest increase in diameter was in the later period of gestation.

The number of myotubes and myofibres per unit area at various CRL is shown graphically in Fig. 8 for all animals studied. The decrease in the number of myotubes is quite clear from 10 · 0 cm. to none at about 37 · 0 cm. (about 155 days gestation according to EVANS/SACK, 1973). This is about halfway through the bovine gestation period which is the same stage at which myotubes appear to be lost in foetal sheep (SWATLAND/CASSENS, 1973 a). At about the same time as the myotube appearance is lost, the number of myofibres (per unit area) decreases (Fig. 8). Up to this stage (about 35 cm.) the number (per unit area) has been increasing as intercellular space becomes less (Figs. 3, 4), but beyond this point the number (per unit area) decreases due mainly to the large increase in diameter (Fig. 7), although intercellular space is also reduced (Figs. 3, 4).

The number of nuclei (per unit area) (Fig. 8) appears to show no significant change over the period studied. It can be seen, however, that the "nuclei" points in this figure show a considerable spread in the pre-40 cm. CRL foetuses. This large spread may be obscuring a significant decline in number of nuclei which might be seen if more foetuses were sampled. This possibility should not be ruled out, especially as STICKLAND et al., (1975) working on pigs and MONTGOMERY et al., (1964) working on the fowl, found that concentration of nuclei was highest in animals with a low body weight.

Fig. 9 is a theoretical figure worked out from the values of Fig. 2 and lines 1, 3, 4 and 5 of Fig. 8. It is intended to give an indication of how the relative numbers of myotubes, myofibres and nuclei in a whole muscle

cross-section change with age of the foetus. A combination of Figs. 2 and 8 assumes, of course, that the trend of cross-sectional muscle area growth in *m. longissimus dorsi* is the same as *m. peroneus longus*. From Joubert's (1956) work, this would seem a reasonable assumption and, although there may be some difference in the actual value of the regression coefficient, the general trends would be the same as Fig. 9. This figure indicates that the total number of myotubes remains at a low figure until about 37 cm. when there are no myotubes. Myofibre number follows a similar shaped curve to muscle cross-sectional area growth (Fig. 2) but increasing to a lesser extent from about 37 cm. onwards. This seems to correspond to the stage when the myofibres start to show their greatest diameter increase, so that from this stage (37 cm.) it is possible that cell size increase gradually takes over from number increase in contributing to increase in muscle cross-sectional area. However, the diameter increases shown in Fig. 7, even in the later period of gestation, are still probably not great enough to account for the very large increase in muscle cross-sectional area (Fig. 2), e. g. the increase in cross-sectional area from 55 to 65 cm. CRL of over 100 % would mean an increase in myofibre diameter from $5 \cdot 6\ \mu m$ (area of $24 \cdot 5\ \mu m^2$) to $7 \cdot 9\ \mu m$ (area of $49 \cdot 0\ \mu m^2$) if there were no number increase. This sort of diameter increase does not occur (Fig. 7). However, although there is an apparent large increase of myofibre number in the later part of gestation, this is probably due mainly to an increase in the length of existing myofibres. This was first suggested by MacCallum (1898) and discussed further by Swatland/Cassens (1972). Many authors, including Joubert (1956) and Swatland/Cassens (1973 a), have suggested that hyperplasia has been completed by two-thirds of the gestation period. In a study such as this it is obviously very difficult to assess when hyperplasia has ceased. It is evident, however, that between $56 \cdot 0$ and $63 \cdot 0$ cm. the incidence of small diameter myofibres drops quite considerably (Fig. 7), which may be an indication that no new fibres are being formed.

As far as the nuclei are concerned in this study, the total number (Fig. 9) understandably follows the same curve as increase in muscle area (Fig. 2). Montgomery (1962) and Cheek (1968) also found the number of nuclei increased with muscle growth in man.

In conclusion it may be said that muscle development in the bovine foetus is similar, from a histological point of view, to the development of muscle in other agricultural animals so far studied, except perhaps for the fact that the myotubes and myofibres appear to be more randomly arranged. The most important aspect of this work, however, is the quantification of the changes in various cell parameters that take place during normal muscle development and the estimation of the time-sequence of these events.

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Summary

The gross and cellular changes which take place in developing bovine muscle were quantified using 36 Zebu foetuses *(Bos indicus)*, ranging from about 75 to 245 days gestation. The change in weight and cross-sectional area of *m. peroneus longus* exhibited logarithmic growth curves. In transverse sections of *m. longissimus dorsi*, no myotubes were seen after about 35 cm. crown-rump length, at which stage the number (per unit area) of myofibres

started to decrease, after their initial considerable increase. The number (per unit area) of nuclei showed no significant change, indicating a direct relationship between number of nuclei and muscle size. The mean diameter of myotubes and, to a lesser extent, myofibres decreased up to the 35 cm. stage, but after this the myofibres clearly increased in diameter. At 60 cm. there was a marked reduction in the number of small diameter myofibres which may indicate that no new myofibres are formed after this stage. The overall results suggest that the increase in muscle cross-sectional area, especially in later gestation, is greater than can be accounted for by increased myofibre diameter alone. It seems likely, in later stages, that the increase in the length of existing myofibres makes a considerable contribution to the increase in muscle diameter.

Zusammenfassung

Eine quantitative Studie der Muskelentwicklung beim Rinderfetus (Bos indicus)

Makroskopische und zelluläre Veränderungen im sich entwickelnden Muskel des Rindes wurden bei 36 Zebufeten im Alter von 75—245 Tagen quantitativ bestimmt. Die Gewichts- und Querschnittsveränderung des M. peronaeus longus weist logarithmische Wachstumskurven auf. In Querschnitten des M. longissimus dorsi wurden beim 35 cm langen Fetus keine Myotubi gefunden. Zu diesem Zeitpunkt beginnt die Anzahl der Muskelfasern (pro Flächeneinheit) abzunehmen, nachdem sie zuvor beträchtlich zugenommen hatte. Die Anzahl der Zellkerne (pro Flächeneinheit) veränderte sich nicht auffällig, was eine direkte Beziehung zwischen Kernanzahl und Muskelgröße anzeigt. Der mittlere Durchmesser der Myotubi und, in geringerem Maße, der Muskelfasern nahm bis zu einer Fetuslänge von 35 cm ständig ab, danach aber nahm der Muskelfaserquerschnitt deutlich zu. Bei 60 cm Körperlänge war die Anzahl von Muskelfasern mit kleinem Durchmesser auffallend verringert, was bedeuten kann, daß nach diesem Stadium keine neuen Muskelfasern gebildet werden. Das Gesamtergebnis läßt den Schluß zu, daß (besonders bei fortgeschrittener Trächtigkeit) die Muskelquerschnittsfläche stärker zunimmt, als sie durch Zunahme des Muskelfaserquerschnitts allein erklärt werden könnte. In späteren Stadien scheint die Längenzunahme der vorhandenen Muskelfasern erheblich zur Vergrößerung des Muskelquerschnitts beizutragen.

Résumé

Recherches quantitatives sur le développement du tissue musculaire chez le foetus bovin (Bos indicus)

On a mesuré les changements qui surviennent au cours du développement du tissu musculaire sur 36 foetus de Zebu (Bos indicus) échelonnés de 75 à 245 jours de gestation. L'évolution du poids et de la surface de section transversale du muscle peroneus longus a présenté une courbe de croissance logarithmique. Sur les sections transversales du muscle longissimus dorsi on n'a pas rencontré de myotubes après le stade d'environ 35 cm de longueur vertex-coccyx. A ce moment, le nombre de fibres musculaires par unité de surface commence à diminuer, alors qu'il avait considérablement augmenté initialement. Le nombre de noyaux par unité de surface n'a pas varié de façon significative, indiquant ainsi une relation directe entre le nombre de noyaux et la

dimension du muscle. Le diamètre moyen des myotubes et jusqu'à un certain point des fibres musculaires a diminué jusqu'au stade de 35 cm, après lequel le diamètre des fibres musculaires s'est accru de façon nette. A 60 cm on a noté une réduction marquée du nombre de fibres musculaires de petit diamètre ce qui pourrait indiquer la formation de nouvelles fibres après ce stade. Le résultat global suggère que l'accroissement de la surface de section transversale du muscle, spécialement dans les derniers temps de la gestation, est plus grand que ce que pourrait laisser attendre la seule augmentation du diamètre des fibres musculaires. Il semble vraisemblable qu'à cette époque, l'accroissement de longueur des fibres musculaires existantes contribue de façon importante à l'augmentation du diamètre du muscle.

Resumen

Un estudio cuantitativo sobre el desarrollo muscular en el feto bovine (Bos indicus)

En 36 fetos del cebú, entre 75 y 245 días de edad, se estudiaron los cambios macroscópicos y celulares durante el desarrollo del músculo bovino en forma cuantitativa. Los cambios del peso y de las áreas de cortes transversales del M. peronaeus longus mostraron curvas logarítmicas de crecimiento. En cortes transversales del M. longissimus dorsi no se encontraron miotúbulos en fetos de 35 cm. de longitud. En esta época, en número de miofibrillas (por unidad del área) comienza a reducirse, después de un aumento inicial considerable. El número de núcleos (por unidad del área) no cambió significativamente, que indica una relación directa entre número de núcleos y tamaño del músculo. El promedio de los diámetros de los miotúbulos, y en menor grado también de las miofibrillas, decreció hasta la etapa de 35 cm., pero luego el diámetro de las miofibrillas aumentó claramente. A los 60 cm., se observó una reducción marcada del número de miofibrillas de pequeño diámetro, lo cual indica que no se forman nuevas miofibrillas después de esta etapa. En general, los resultados indican que el aumento del área de cortes transversales del músculo (especialmente en etapas tardías de la gestación) es mayor que los valores deducidos del incremento del diámetro de las miofibrillas sólo. En etapas ulteriores, parece verosímil que el aumento de la longitud de las miofibrillas existentes contribuye considerablemente al aumento del diámetro muscular.

References

Ashmore, C. R., D. W. Robinson, P. Rattray, and L. Doerr, 1972: Biphasic development of muscle fibers in the fetal lamb. Exp. Neurol. *37*, 241.

Ashmore, C. R., P. B. Addis, and L. Doerr, 1973: Development of muscle fibres in the fetal pig. J. Anim. Sci. *36*, 1088.

Cheek, D. B., 1968: Human Growth. Lea & Febiger, Philadelphia.

Danilova, L. V., 1960: The development of the hind limb musculature in cattle, Bos taurus. Trudy Inst. Morfol. Zhivot. Akad. Nauk S.S.S.R. *29*, 34.

Eliot, T. S., R. C. Wigginton, and K. B. Corbin, 1943: The number and size of muscle fibers in the rat soleus in relation to age, sex and exercise. Anat. Rec. *85*, 307.

Evans, H. E., and W. O. Sack, 1973: Prenatal development of domestic and laboratory mammals. Growth curves, external features and selected references. Zbl. Vet. Med. C, Anat., Histol., Embryol., *2*, 11.

Goldspink, G., 1964: The combined effects of exercise and reduced food intake on skeletal muscle fibers. J. Cell. Comp. Physiol. *63*, 209.

Hammond, J., and A. B. Appleton, 1932: Study of the Leg of Mutton. Part V. Growth and Development of Mutton Qualities in the Sheep. Oliver & Boyd, London.

Holtzer, H., and R. Bischoff, 1970: Mitosis and myogenesis. In: The Physiology and Biochemistry of Muscle as a Food, 2. E. J. Briskey, R. G. Cassens, and B. B. Marsh (Ed.), The University of Wisconsin Press, Madison and London.

JOUBERT, D. M., 1955: Growth of muscle fibre in the foetal sheep. Nature *175*, 936.
JOUBERT, D. M., 1956: A study of pre-natal growth and development in the sheep. J. Agric. Sci. *47*, 382.
KELLEY, A. M., and S. I. ZACKS, 1969: The histogenesis of rat intercostal muscle. J. Cell Biol. *42*, 135.
KIKUCHI, T., 1971: Studies on the development and differentiation of muscle. III. Especially on the mode of increase in the number of cells. Tohuku J. Agric. Res. *22*, 1.
MACCALLUM, J. B., 1898: On the histogenesis of the striated muscle fibre and the growth of the human sartorius muscle. Johns Hopkins Hosp. Bull. *9*, 208.
MONTGOMERY, R. D., 1962: Growth of human striated muscle. Nature *195*, 194.
MONTGOMERY, R. D., J. W. T. DICKERSON, and R. A. MCCANCE, 1964: Severe undernutrition in growing and adult animals. 13. The morphology and chemistry of development and undernutrition in the sartorius muscle of the fowl. Br. J. Nutr. *18*, 587.
OMMER, P. A., 1971: Histochemical differentiation of skeletal muscle fibres in the bovine foetus. Experientia *27*, 173.
ROWE, R. W. D., and G. GOLDSPINK, 1969: Muscle fibre growth in five different muscles in both sexes of mice. I. Normal mice. J. Anat. *104*, 519.
SNEDECOR, G. W., and W. G. COCHRAN, 1967: Statistical Methods. 6th Edition. The Iowa State University Press, Ames, Iowa.
STAUN, H., 1963: Various factors affecting number and size of muscle fibres in the pig. Acta Agric. Scand. *XIII*, 293.
STAUN, H., 1968: Diameter and number of muscle fibres and their relation to meatiness and meat quality in Danish Landrace pigs. 366. beretn. fra forsøgslab. København.
STICKLAND, N. C., 1975: A detailed analysis of the effects of various fixatives on animal tissue with particular reference to muscle tissue. Stain Tech. *50*, 255.
STICKLAND, N. C., and G. GOLDSPINK, 1973: A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. Anim. Prod. *16*, 135.
STICKLAND, N. C., and G. GOLDSPINK, 1975: A note on porcine skeletal muscle parameters and their possible use in early progeny testing. Anim. Prod. *21*, 93.
STICKLAND, N. C., E. M. WIDDOWSON, and G. GOLDSPINK, 1975: Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscle of pigs. Br. J. Nutr. *34*, 421.
SWATLAND, H. J., and R. G. CASSENS, 1972: Muscle growth: the problem of muscle fibers with an intrafascicular termination. J. Anim. Sci. *35*, 336.
SWATLAND, H. J., and R. G. CASSENS, 1973: Prenatal development, histochemistry and innervation of porcine muscle. J. Anim. Sci. *36*, 343.
SWATLAND, H. J., and R. G. CASSENS, 1973 a: Inhibition of muscle growth in foetal sheep. J. Agric. Sci. *80*, 503.
THURLEY, D. C., 1972: Increase in diameter of muscle fibres in the foetal pig. Br. Vet. J. *128*, 355.
WINTERS, L. M., W. W. GREEN, and R. E. COMSTOCK, 1942: Prenatal development of the bovine. Techn. Bull. Univ. Minn. Agric. Exp. Sta. *151*, 1.
WOHLFART, G., 1937: Über das Vorkommen verschiedener Arten von Muskelfasern in der Skelettmuskulatur des Menschen und einiger Säugetiere. Acta Psychiat. et Neurol. Supp. *12*, 1.

Author's address: Dr. N. C. STICKLAND, Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH, U.K.

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Muscle development in the human fetus as exemplified by m. sartorius: a quantitative study

N. C. STICKLAND

Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH

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INTRODUCTION

The size of an individual muscle is determined by the number and size of its constituent muscle fibres and, to a lesser extent, by the amount of connective tissue. It is known from several studies on both laboratory animals and agricultural animals that the size of muscle fibres can be affected by many factors such as age (Hammond & Appleton, 1932; Meara, 1947; Joubert, 1956*a*; Goldspink, 1962; Stickland & Goldspink, 1973), exercise (Goldspink, 1964) and level of nutrition (Joubert, 1956*a*; Stickland, Widdowson & Goldspink, 1975). The number of muscle fibres, on the other hand, appears to be genetically determined, showing no significant change after birth in most animals studied including the pig (Staun, 1963; Stickland & Goldspink, 1973) and certain laboratory animals (Eliot, Wigginton & Corbin, 1943; Rowe & Goldspink, 1969).

It is evident that the number of muscle fibres in a given muscle increases before birth until the genetically determined number is attained. The time during gestation when hyperplasia ceases has seemingly not been investigated in any detail except for a few studies including those on sheep by Joubert (1955, 1956*b*) and Swatland & Cassens (1973*a*), and on the ox by Stickland (1978). It is very often stated, however, that hyperplasia in human muscle is completed some time before birth. This statement is based on either the extensively quoted work of MacCallum (1898) who used muscle fibre counts from m. sartorius of each of only five fetuses and one full term baby, or the work of Montgomery (1962) who also used m. sartorius but from only two fetuses and one full term baby. It could well be misleading to try to draw too many conclusions from such limited data, especially when it is appreciated how large is the adult variation in fibre number in the muscles used, as shown by the results of these authors on several adult muscles.

Most work on human skeletal muscle has, in fact, been concerned with descriptions of the various muscular disorders (e.g. Adams, 1975). It is often stated by muscle pathologists that a large number of muscle diseases may be caused by various abnormalities of muscle development *in utero*. One example is myotubular myopathy in which, according to Spiro, Shy & Gonatas (1966), there is persistence of fetal myotubes in extrauterine life. Although myotubes appear in normal neonatal rat muscle (Ontell, 1977) they are an abnormal phenomenon in neonatal human muscle. This stresses one of the reasons why comparisons between animal and human muscle can often be misleading. Apart from studies on muscle diseases there have also been various descriptive or qualitative approaches to prenatal muscle development in the human, from the light microscopic study of Hewer (1927) to the ultrastructural

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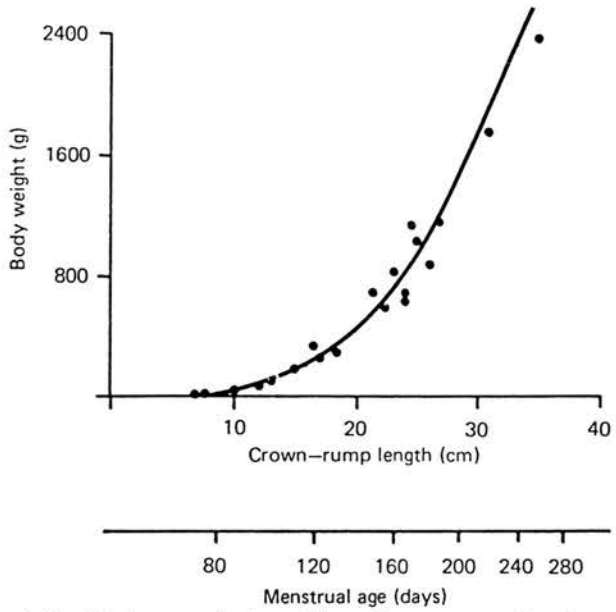

Fig. 1. The relationship between body weight and crown–rump length, and an indication of menstrual age (280 days = birth).

study of Tomanek & Colling-Saltin (1977). There has, however, been no comprehensive quantitative study of prenatal human muscle development based on an adequately large number of fetuses.

A study which defined, in quantitative terms, the pattern of normal muscle development in the human would be of interest to the pathologist studying developmental myopathies which could then be more clearly defined in quantitative terms.

The purpose of this investigation was, therefore, to quantify both the gross and cellular changes (including nuclei numbers, muscle cell size and numbers and myofibril proliferation) which take place in muscle development, but paying particular attention to the relative contributions of hyperplasia and hypertrophy to prenatal muscle growth.

MATERIALS AND METHODS

Muscle samples were removed from 21 fetuses which ranged in crown–rump length (CR) from 7·0 to 35·0 cm, which approximately corresponds to a range in menstrual age from 80 to 270 days (i.e. to about 10 days before birth: from the data cited by Langman, 1975). In order to overcome possible errors, it was decided to discuss the parameters investigated in relation to CR rather than an estimated age. CR may also be a more relevant parameter than age in this particular study as it is known that general growth is more related to the physiological age of an animal than the chronological age (Ragsdale, 1934). The weight of a fetus, although probably not so related to age as CR, may possibly be more closely related to some features of muscle development than CR and, therefore, another useful fetus parameter to consider in certain discussions. The fresh body weight (BW) and CR of each fetus used (together with an estimation of age) are shown graphically in Figure 1 which shows that, as one might expect, BW is approximately proportional to $(CR)^3$.

M. sartorius was removed entire from a hind limb of each fetus. The smaller fetuses had all been immersed in phosphate-buffered (pH 7·4) formalin solution so that the muscles could be removed fixed *in situ*. The muscles had to be removed fresh from the two largest fetuses and splinted at the *in situ* length (flexed limb position) before fixation. After this initial fixation, the length and weight of each muscle was noted. Complete transverse sections (2 mm thick) were then cut from each muscle at its mid-length level. These muscle sections (the larger ones cut into two or three segments longitudinally) were then placed in a phosphate-buffered (pH 7·4) paraformaldehyde–glutaraldehyde mixture (Karnovsky, 1965) for several hours before washing in phosphate buffer, post-fixation in 1 % osmium tetroxide and further washing. Dehydration was then carried out through a series of alcohols from 10 % to absolute, followed by clearing in propylene oxide and finally infiltration by, firstly, a mixture of propylene oxide and Araldite and then in Araldite alone, in which the tissue samples were also embedded. Transverse sections (1·5 μm thick) were cut from each block (with a few also cut longitudinally) on a Reichert OMU3 ultramicrotome using glass knives, and stained with 0·5 % toluidine blue.

Quantitative histology

The sections were first viewed under low power magnification and the image projected on to a screen so that the total section could be outlined thereby enabling total muscle cross sectional area to be measured. The sections were then viewed with a Leitz Ortholux microscope under $\times 40$ and $\times 100$ (oil immersion) objectives and several random photomicrographs obtained using a Leitz Orthomat camera attachment.

Numbers of cells and nuclei

The photomicrographs were used to estimate the numbers of muscle cells and nuclei per unit area. The area of the $\times 100$ objective photomicrograph was 0·0054 mm^2 and this was taken as the unit area. The $\times 40$ photomicrographs were used for the larger fetuses in order to cover a larger sampling area but not for the smaller fetuses, as cells in these were sometimes difficult to distinguish with this lower magnification. The results for the $\times 40$ photomicrographs were always checked for accuracy by comparing counts from a few $\times 100$ photomicrographs. On average, sufficient measurements were made so that cells in about 5 % of the total cross sectional area had been counted. The cells counted were classified as myotubes if myofibrils were peripheral with a clear area or nucleus in the centre, or myofibres if the myofibrils filled the cell and nuclei, if present, were peripheral. All nuclei were counted, no effort being made to distinguish satellite and other cell nuclei from muscle cell nuclei, as this was felt to be too difficult for accurate estimation at the light microscope level. However, there was the added advantage that direct comparisons could be made with biochemical estimations of DNA in developing muscle.

The total number of myofibres, myotubes and nuclei in the complete section of each muscle was estimated from the results of numbers per unit area and the total muscle cross sectional area measurements.

An estimation was made of the total number of nuclei in the complete m. sartorius of each fetus. This estimation was based upon the relationship derived by Abercrombie (1946), namely $N = (T/[T+D])\,n$, where N = the real number of nuclei whose centres are included in the section, n = the number of apparent nuclei

counted, T = the section thickness (1·5 μm), and D = the mean longitudinal dimension of the nucleus. The maximum nuclear length seen in the longitudinal sections which were made was found to be about 9·3 μm and was taken as the value of D. In this investigation, therefore, $N = 0{\cdot}1389\,n$. To obtain the total number of nuclei in each muscle this figure was then multiplied by $L/1{\cdot}5$, where L = muscle length (in μm) and 1·5 is the section thickness (in μm).

Size of cells

For each fetus the cross sectional area of 100 cells of each type (myotubes and myofibres) was measured using a planimeter (Clarkson's Zero-setting Compensating Planimeter) on the photomicrographs obtained with the × 100 objective. When the proportion of myotubes or myofibres was very small it was sometimes possible to measure only 50 cells. The mean cross sectional area of myotubes and myofibres for each fetus was then calculated. From these area measurements, diameters were estimated assuming the cross sectional areas to be circular, i.e. diameter = $\sqrt{([4 \times \text{Area}]/\pi)}$. These diameters were used to create histograms for some of the fetuses (CR 7·0, 7·5, 18·5, 23·0, 27·0 and 35·0 cm). Histograms of cell areas were not used as these give skewed distributions which obliterate many features of interest.

Intercellular space

The amount of intercellular space seen in the sections was calculated by multiplying the numbers of myotubes and myofibres per unit area (0·0054 mm²) by their respective mean cross sectional areas. This gave the area in the cross section occupied by muscle cells (X mm²), the percentage area being $(0{\cdot}0054 - X)/0{\cdot}0054 \times 100$. This figure subtracted from 100 % would, of course, give the percentage of intercellular space, thereby defining it here as that area not occupied by myotubes or myofibres.

Number of myofibrils

For each fetus the number of myofibrils in 10 myofibres and, when present, 10 myotubes was counted using the high power photomicrographs. The number of myofibrils per μm² was then calculated from these counts for each fetus and used to estimate the number of myofibrils in myofibres and, where applicable, myotubes of the mean cross sectional area already estimated (see above). Although, of course, the number of myofibrils per cell is very variable the number per μm² (the figure required) in any given muscle is fairly consistent, so that 10 was considered an adequate number of cells to measure. This was also the number used by Goldspink (1970).

Presentation of data

It was felt that, as the main aim of this investigation was to quantify the changes in various muscle parameters which take place during prenatal growth, the data could be most clearly presented in a graphical form. Most of the muscle parameters investigated were plotted against CR or BW for the reasons already discussed. An approximate indication of age can, however, be ascertained from Figure 1.

An attempt was made to define the plotted relationships by linear regression equations which frequently involved transforming one or both of the parameters, usually to the common logarithm. Various transformations were attempted for each plot until the best fit linear regression was found. When a linear regression did not appear to fit the relationship, however, an estimate curve was drawn, based upon

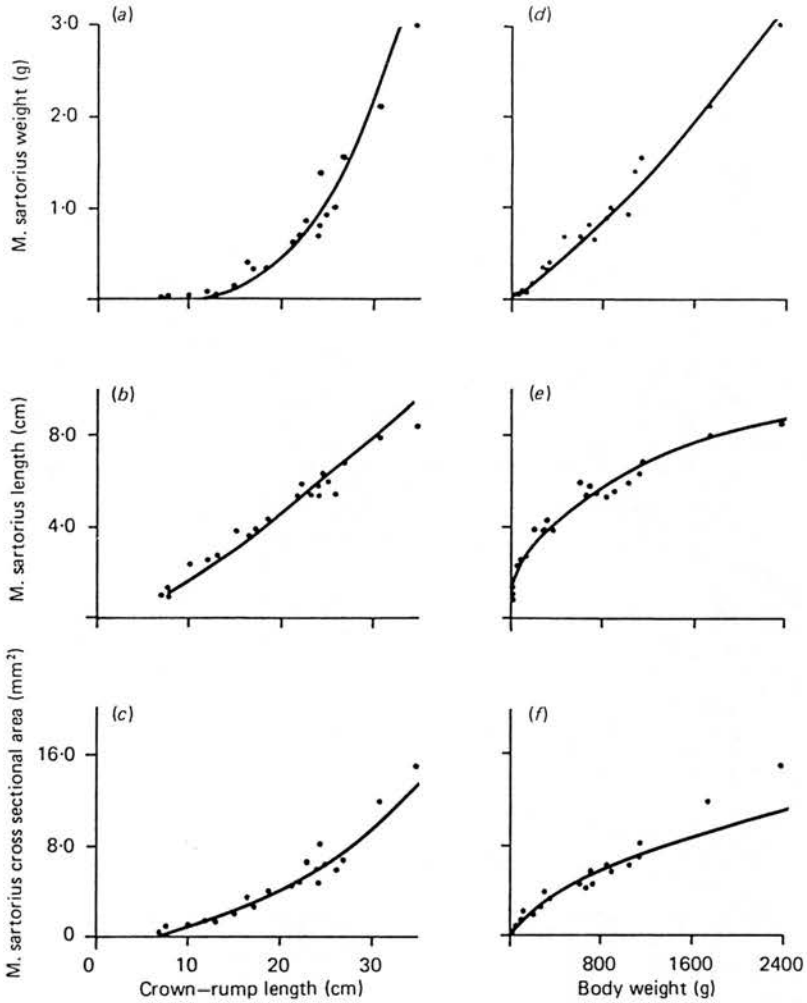

Fig. 2. The relationship between m. sartorius weight (*a*, *d*), length (*b*, *e*), cross sectional area (*c*, *f*) and (*a*, *b*, *c*) crown–rump length and (*d*, *e*, *f*) body weight.

previous computed lines, as described in the following section for the relationships concerned. Statistical methods were based upon Snedecor & Cochran (1967).

RESULTS

Gross parameters

The relationships between the weight, length and cross sectional area (at the mid-length level) of m. sartorius with both CR and BW are shown graphically in Figure 2, the equations of these curves being given in Table 1. This shows that the best fit curve for all these relationships is the power curve or 'allometric' relationship of the form $y = a.X^b$, where b is the differential growth ratio defined by Huxley (1924). This relationship is linear when log Y is plotted against log X (i.e. $\log Y = \log a + b.\log X$). The value of b will be related to the dimensions of the parameters being investigated, e.g. when muscle weight (three dimensional parameter) is plotted

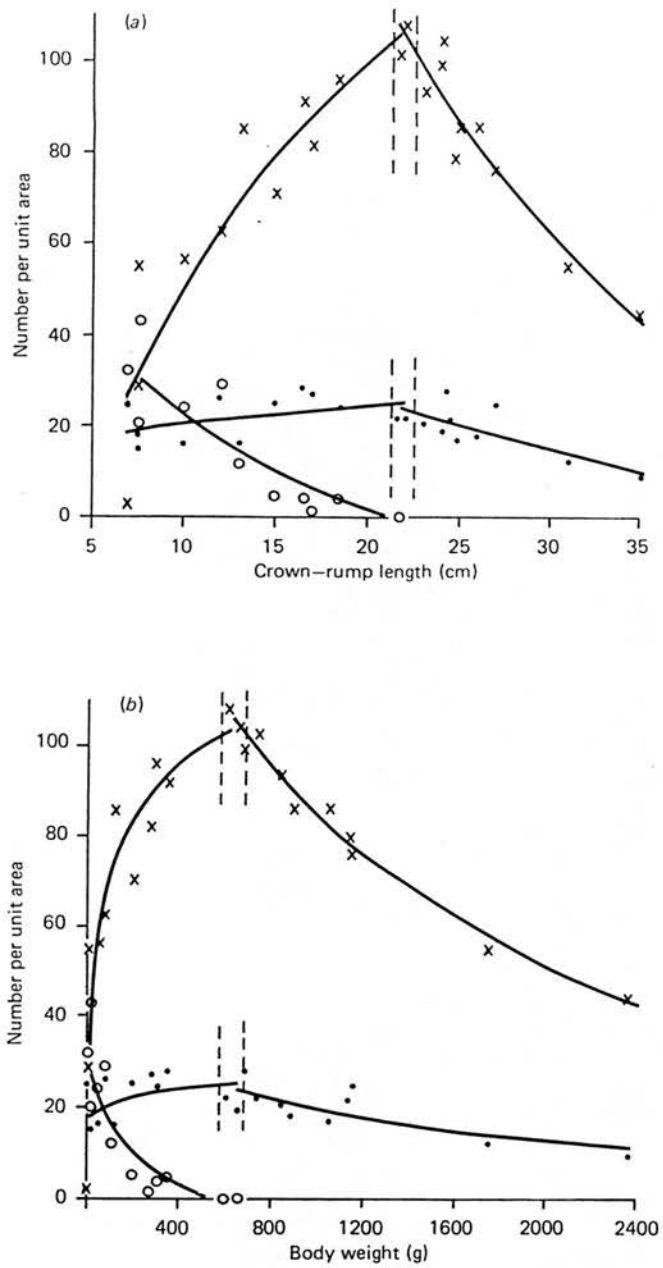

Fig. 3. The relationship between the number of myofibres (×), myotubes (○), and nuclei (●) per unit area and (*a*) crown–rump length, (*b*) body weight.

against CR (one dimensional), the value of *b* would be expected to approximate to 3. The fact that *b* is considerably higher ($P < 0{\cdot}001$) than this, with a value of 3·9 (Table 1), shows that m. sartorius weight has a higher growth rate than CR. Furthermore, the value of *b* in Figure 2*b* is significantly higher ($P < 0{\cdot}001$) than the 'expected' 1·00, whereas *b* in Figure 2*c* is not significantly different from the 'expected' 2·00. Taken as a whole, these results suggest that most of the higher rate

Table 1. *Equations of the computed regression lines shown in the Figures*

Figure	Parameters *X*	Parameters *Y*		Line	S_b
1	CR	BW		$Y = 3{\cdot}13.10^{-2}.X^{3{\cdot}20}$	0·042
2(*a*)	CR	Muscle weight		$Y = 3{\cdot}47.10^{-6}.X^{3{\cdot}91}$	0·162
2(*b*)	CR	Muscle length		$Y = 9{\cdot}21.10^{-1}.X^{1{\cdot}30}$	0·032
2(*c*)	CR	Muscle t.s. area		$Y = 8{\cdot}01.10^{-3}.X^{2{\cdot}07}$	0·064
2(*d*)	BW	Muscle weight		$Y = 2{\cdot}42.10^{-4}.X^{1{\cdot}22}$	0·076
2(*e*)	BW	Muscle length		$Y = 4{\cdot}12.10^{-1}.X^{0{\cdot}39}$	0·009
2(*f*)	BW	Muscle t.s. area		$Y = 9{\cdot}23.10^{-2}.X^{0{\cdot}62}$	0·024
3(*a*)	CR[a](7–22·5)	Fibres	No. per unit area	$Y = 161.\log X - 110$	5·69
	CR (21–35)	Fibres		$Y = 515 - 306.\log X$	5·42
	CR (7–22·5)	Nuclei		$Y = 11{\cdot}8.\log X + 8{\cdot}96$	1·18
	CR (21–35)	Nuclei		$Y = 108 - 62{\cdot}9.\log X$	1·36
	CR	Tubes		$Y = 93{\cdot}7 - 70{\cdot}9.\log X$	4·15
3(*b*)	BW[b](0–680)	Fibres	No. per unit area	$Y = 40{\cdot}4.\log X - 10{\cdot}6$	1·32
	BW (600–2400)	Fibres		$Y = 414 - 110.\log X$	2·70
	BW (0–680)	Nuclei		$Y = 4{\cdot}94.\log X + 10{\cdot}9$	0·70
	BW (600–2400)	Nuclei		$Y = 85{\cdot}1 - 21{\cdot}9.\log X$	0·67
	BW	Tubes		$Y = 61{\cdot}1 - 22{\cdot}5.\log X$	2·42
4(*b*)	BW	Fibres	Total no. per section	$Y = 64685.\log X - 95932$	$2{\cdot}37.10^3$
	BW	Nuclei		$Y = 13649.\log X - 18462$	$0{\cdot}53.10^3$
5(*b*)	BW	Total no. nuclei in whole muscle		$Y = 9{\cdot}38.10^7.\log X - 1{\cdot}53.10^8$	$0{\cdot}42.10^7$
6	$(CR)^2$	Mean fibre area		$Y = 26{\cdot}18.1{\cdot}0009^x$	0·0027
	CR	Mean tube area		$Y = 47{\cdot}23.0{\cdot}9760^x$	0·0931
8	CR	% Cellular area		$Y = 58{\cdot}1.\log X - 10{\cdot}9$	1·47
10	Mean tube area	No. of myofibrils		$Y = 34{\cdot}4 - 0{\cdot}005.X$	0·054
	Mean fibre area	No. of myofibrils		$Y = 106{\cdot}5.\log X - 122{\cdot}0$	1·8

[a] Crown–rump length (cm). [b] Body weight (g).
S_b, Standard error of regression coefficient.

of muscle weight increase relative to CR is due to a higher rate of muscle length increase rather than cross sectional area increase. This situation also seems to apply for these parameters relative to BW (Fig. 2*d*, *e*, *f*).

Numbers of cells and nuclei

The numbers of myofibres, myotubes and nuclei per unit area (0·0054 mm²) of tissue section are plotted against CR in Figure 3*a* and BW in Figure 3*b*. At about 22 cm CR (Fig. 3*a*) and 650 g BW (Fig. 3*b*) there appears to be a very distinct change in the trends of the plotted points. For this reason the plots are divided into two segments at these particular CR and BW values. The equations for all the computed regression lines shown in Figure 3 are given in Table 1. The best curves to define these plots were all found to be logarithmic. Figure 3 shows that there is a considerable increase in the number of fibres per unit area up to about 22 cm CR or 650 g BW with a concomitant decrease in the numbers of myotubes to zero at these points. This means that there is initially a slightly higher proportion of myotubes to myofibres, but this is rapidly reversed. The number of nuclei per unit area increases slightly up to 21 cm CR or 550 g BW and then decreases. There appears to be no clear evidence for a better regression of any of these parameters on either CR or BW (Table 1). The slopes of all the lines shown in Figure 3, including all nuclei number lines, are significantly ($P < 0{\cdot}001$) different from the horizontal.

The total numbers of myofibres, myotubes and nuclei in the complete cross section of each muscle are plotted against CR in Figure 4*a* and BW in Figure 4*b*. The points in Figure 4*a* do not appear to follow a simple curve. It is possible that this curve is sigmoid, but in order to compute such a curve one must assume an upper asymptote which could be unjustified and, in any event, difficult to estimate. The plotted lines are, therefore, estimates based on the computed lines of Figures 2*c* and 3*a*. The points in Figure 4*b*, however, appear to fit reasonably well to a logarithmic curve (shown by broken lines), the equations of which are given in Table 1. The continuous lines shown in Figure 4*b* are lines estimated from Figures 2*f* and 3*b*, i.e. estimated in a similar way to the lines of Figure 4*a*. There seems to be fairly close agreement between computed and estimated lines in Figure 4*b* except for the divergence in myofibre number from 1200 g onwards. This divergence was converted to CR values and is indicated on Figure 4*a* by a broken line. This broken line probably improves the accuracy of the estimated line in Figure 4*a*, and further support for this adjustment is given later. Taken as a whole, the results in Figure 4 suggest that there is an initial slow increase in the number of myofibres up to about 12 cm CR (not identifiable in Figure 4*b*), followed by a very rapid increase up to about 22·5 cm CR or 700 g BW, after which the number increases at a declining rate. The number of myotubes remains relatively low until decreasing to zero at about 21 cm CR or 550 g BW. The nuclei number increases to about the 25 cm CR or 600 g BW stage and then appears to level off.

The total number of nuclei in whole muscles is plotted against CR in Figure 5*a* and against BW in Figure 5*b*. The lines shown are estimated from Figures 2*b* and 4*a* for Figure 5*a*, and from Figures 2*c* and 4*b* (estimated line) for Figure 5*b*. This is with the exception of the broken line in Figure 5*b* which is a logarithmic curve, whose equation is given in Table 1 and which roughly approximates to the estimated line. It can be seen from Figure 5*a* that there is an initial slow increase of nuclei up to about 15 cm CR, then a more rapid increase which probably, though not significantly, gradually slows down from about 30 cm CR. There would appear to be only slight evidence, therefore, of a slowing down of nuclear increase in later gestation as defined by CR. This is in contrast to Figure 5*b*, however, which shows an initial rapid increase in nuclei followed by a distinct slowing down of nuclear proliferation from about 400 and 800 g BW in computed and estimated curves respectively.

Size of cells

The average cross sectional areas of myotubes and myofibres at each CR are shown in Figure 6. Exponential curves, whose equations are given in Table 1, appear to best describe the decrease in myotube size and increase in myofibre size. It can be seen that at 7·5 cm CR the myotubes are more than 1½ times bigger in cross sectional area than the myofibres, but after this there is a decrease in myotube size and increase in myofibre size until about 15 cm CR when they are similar in size. The myotubes show a further slight decrease in size before they are no longer evident. The myofibres increase in size slowly at first and then more rapidly. The broken line in Figure 6 shows the overall mean size of cells at each CR. This line was estimated by using the computed lines in this figure and the proportions of myotubes and myofibres shown in Figure 3*a*. This shows that there is an initial decrease in overall cell cross sectional area from 7 to 10 cm CR, and from 10 cm onwards there is an initial slow rate of increase which continuously increases to a higher rate

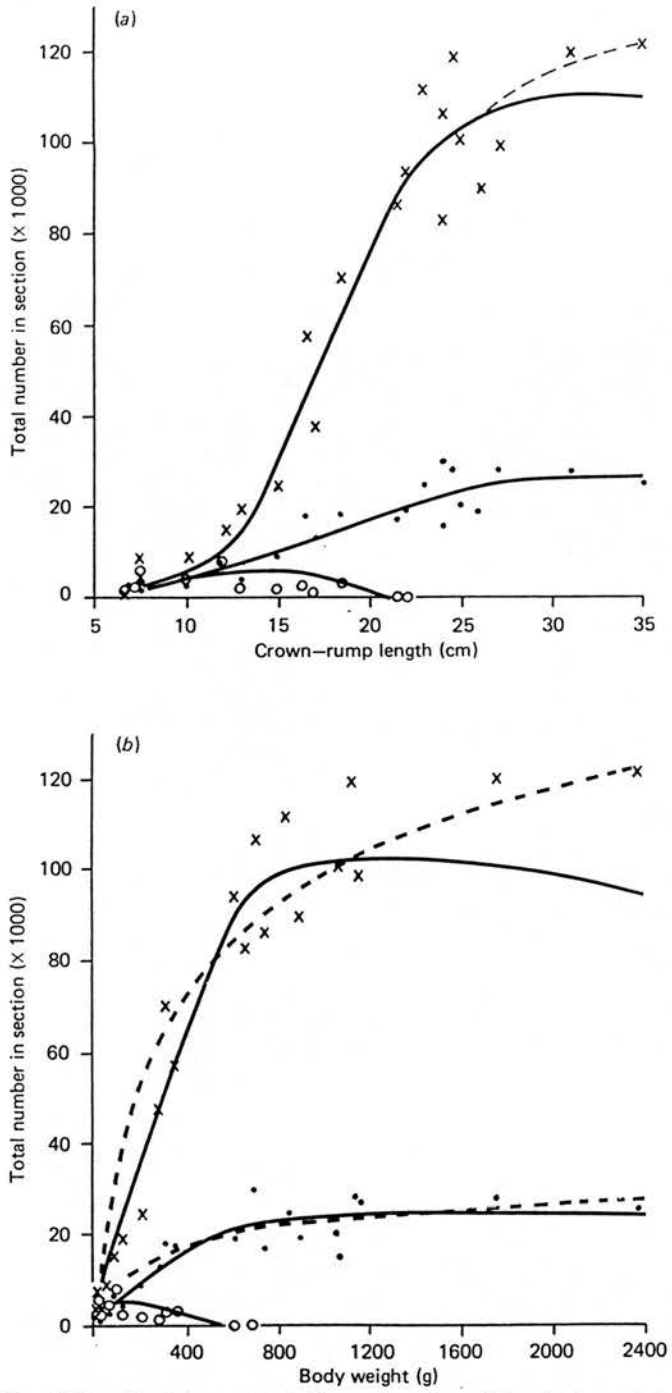

Fig. 4. The relationship between the total number of myofibres (×), myotubes (○) and nuclei (●) in a transverse section of m. sartorius and (*a*) crown–rump length, (*b*) body weight. The continuous lines (——) are estimated from other curves; the broken lines (– – –) are computed from the data points shown.

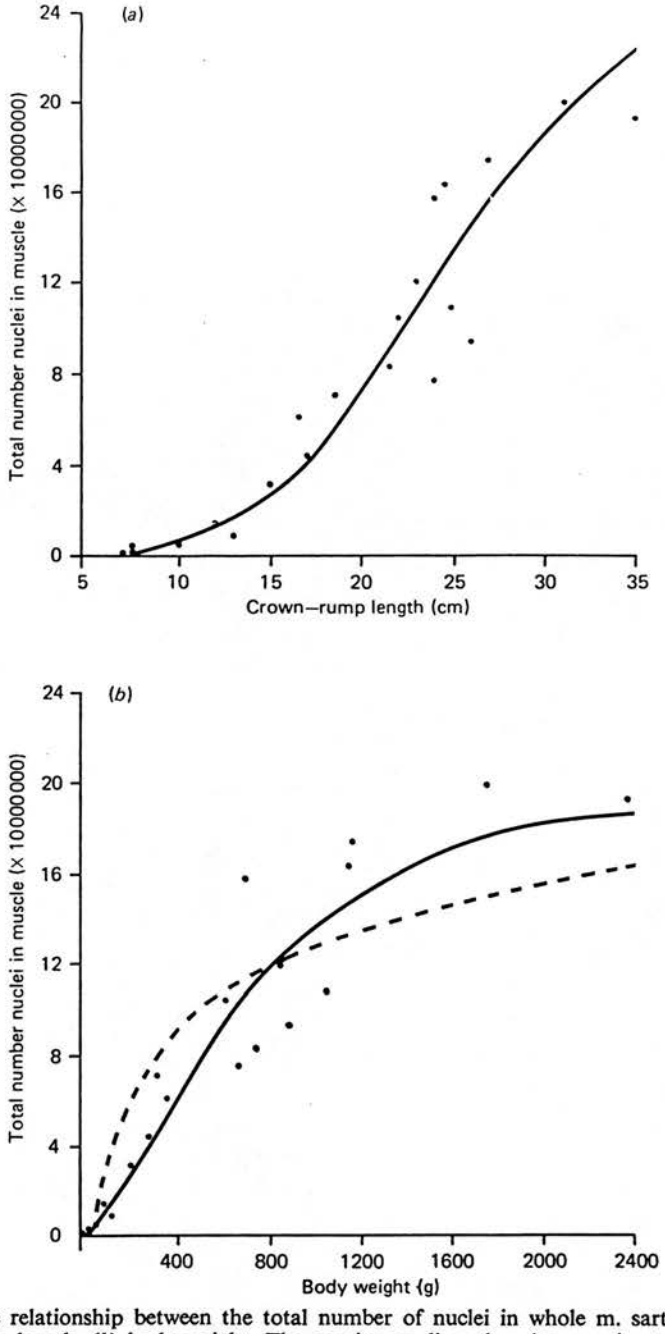

Fig. 5. The relationship between the total number of nuclei in whole m. sartorius and (*a*) crown–rump length, (*b*) body weight. The continuous lines (——) are estimated from other curves; the broken lines (– – –) are computed from the data points shown.

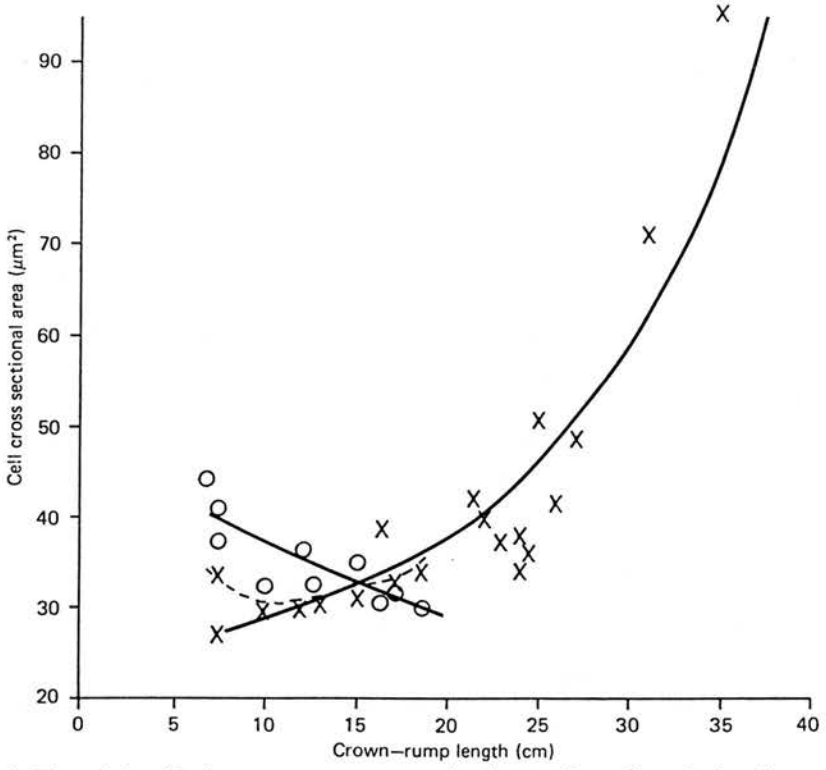

Fig. 6. The relationship between mean cross sectional area of myofibres (×) and myotubes (○) and crown–rump length. The broken line shows the change in overall mean cell cross sectional area.

in later gestation. The change in rate is so marked that, whereas from 10 cm CR to 20 cm CR cross sectional area only increases by about one-fifth, from 10 cm CR to 35 cm CR the area is almost tripled.

Fibre diameter frequency distributions are shown in Figure 7 for the muscles of six fetuses. Histograms such as these were made for all 21 fetuses, but only these six are necessary to demonstrate the general trends. The total proportion of myofibres to myotubes seen in this Figure was based on the cell number counts already mentioned, so that the percentage of these proportions in each size class could be used in the histograms. Figure 7 shows that originally (7·0 cm CR), there is a wide range in the diameter of myotubes from about 6 to 11 μm. There is then (illustrated here by the 7·5 cm CR histogram) a decrease in the frequency of the larger diameter myotubes although the overall range of myotube diameters is similar. At this same stage there is an appearance of myofibres ranging from about 2 to 11 μm in diameter. Later (18·5 cm CR) only a few small diameter myotubes remain (about 6 to 7 μm), although the distribution of myofibres is similar except that there are no myofibres in the smallest size class, i.e. about 2 to 3 μm. The next stage (23·0 cm CR) shows a similar range of myofibre diameters from about 3 to 11 μm, with apparently only the medium diameter fibres increasing in size from the previous (18·5 cm CR) stage. There then (27·0 cm CR) appears to be an increase in size of small, medium and large diameter fibres with an extended upper limit of about 14 μm, although the lower limit remains at about 3 μm. The trend of a general increase is continued

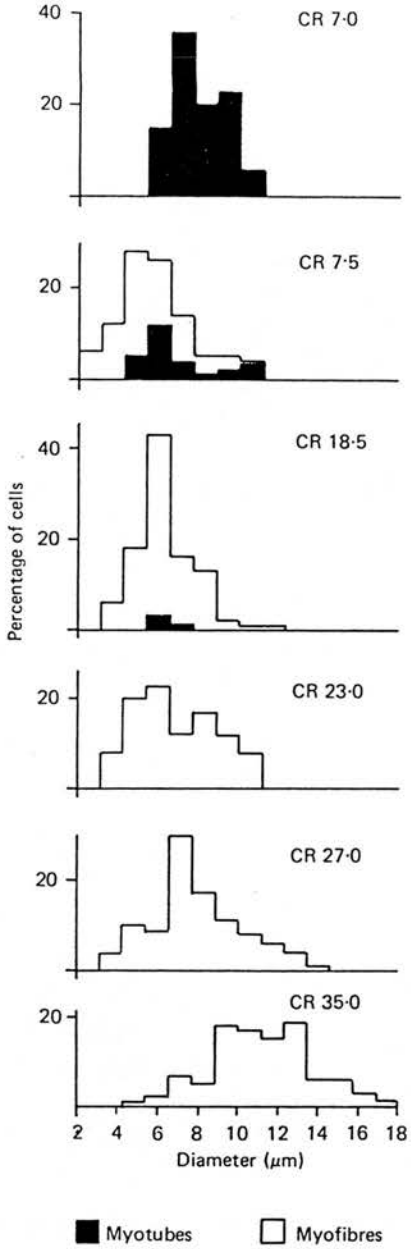

Fig. 7. Histograms showing the distribution of myotube and myofibre diameters at various crown–rump lengths (CR) from 7·0 cm to 35·0 cm.

(35·0 cm CR) with a complete shift in the range of fibre diameters to between 5 and 18 μm.

Intercellular space

The amount of cross sectional area occupied by myotubes and myofibres in each muscle section is plotted abainst CR in Figure 8. This shows that there is an increase (the rate of which steadily decreases) in total cellular area from about 38 % at

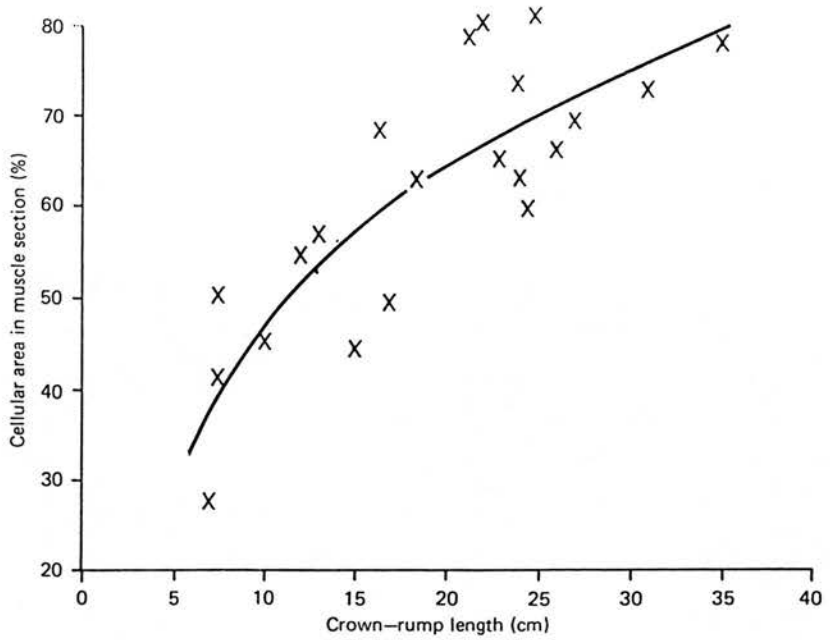

Fig. 8. The relationship between the percentage cellular area in transverse sections of m. sartorius and crown–rump length.

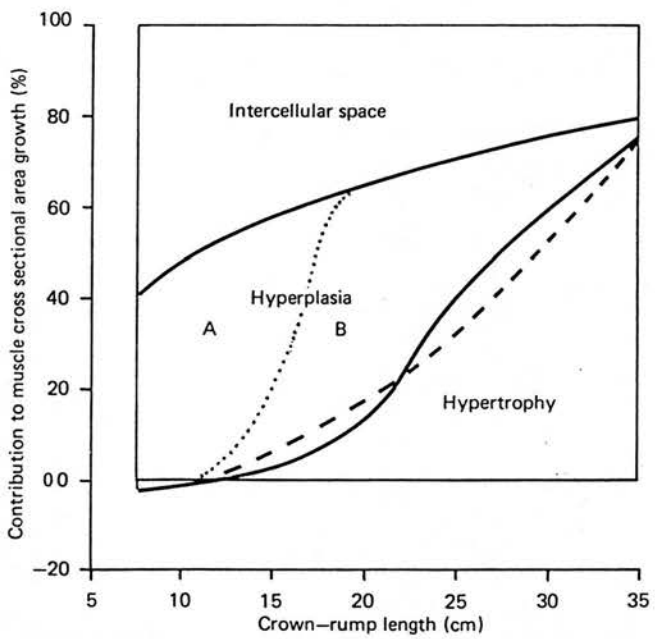

Fig. 9. The change in percentage contributions of hyperplasia and hypertrophy to muscle cross sectional area growth with increasing crown–rump length. The broken line (– – –) and dotted line (.....) are discussed in the text. A is real hyperplasia; B is apparent hyperplasia or longitudinal hypertrophy.

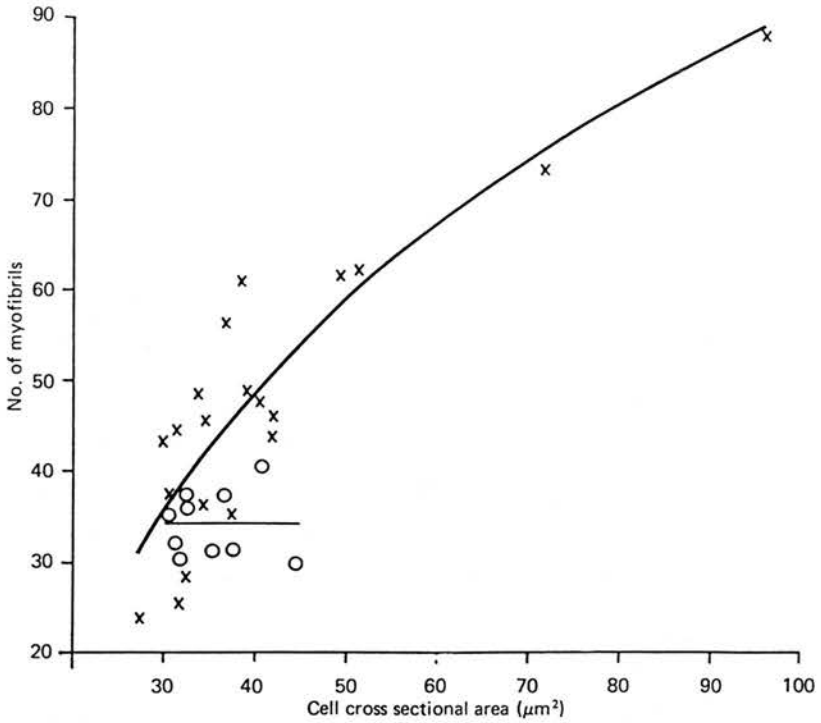

Fig. 10. The relationship between the number of myofibrils and the cross sectional area of myofibres (×) and myotubes (○).

7 cm CR to about 79 % at 35·0 cm CR, i.e. the cellular area doubles between 7·0 and 35·0 cm CR. Conversely, the amount of 'intercellular' space decreases by two thirds, from about 62 % at 7·0 cm CR to about 21 % at 35·0 cm CR. The equation for the computed line in Figure 8 is logarithmic and is given in Table 1.

Relative contributions of hyperplasia and hypertrophy to muscle growth

Figure 9 is an attempt to estimate the relative contributions of hyperplasia and hypertrophy to prenatal muscle cross sectional area growth.

The proportion of intercellular space decreases during the period studied (Fig. 8), which means that the increase in actual cellular cross sectional area is relatively larger than the total muscle cross sectional area. The relative contributions of hyperplasia and hypertrophy to this cellular area increase were estimated by measuring the increase in fibre number (From Fig. 4*a* – including broken line) and mean cell cross sectional area (from Fig. 6) respectively, over defined 2·5 cm CR stages. One type of increase was then expressed as a percentage of the two combined increases and plotted as shown in Figure 9. As a check on the accuracy of the fibre number curves shown in Figure 4*a*, it was decided to use the increases in mean cell cross sectional area and the increases in total cellular area (estimated from a combination of Figures 2*c* and 8) in order to estimate the contribution of hyperplasia by an indirect method. Using these estimated hyperplasia values and the actual hypertrophy values produces the broken line shown in Figure 9. This shows fairly close approximation to the actual relative contributions of hyperplasia and hypertrophy to muscle cross sectional area growth. The results of Figure 9 show very clearly that

there is a marked change from hyperplasia as the only positive contribution to muscle cellular cross sectional area growth early on, to a 50:50 contribution with hypertrophy at about 23·5 cm CR, and then to only about a 6% contribution (hypertrophy 94%) at 35·0 cm CR. Figure 9 is discussed at greater length later.

Number of myofibrils

The numbers of myofibrils in mean-sized myotubes and myofibres for each fetus are plotted against cell cross sectional area in Figure 10. The equations for each line are given in Table 1. The curve for myofibres is logarithmic, indicating that the number of myofibrils does not quite keep pace with increase in cross sectional area of the myofibre. There was no evidence, however, for a relationship between size of myotubes and myofibril number, and in fact there was no significant difference from the horizontal for the regression line shown.

DISCUSSION

Gross aspects

The relationship between CR and BW is within the normal range exhibited by the human fetus according to the figures of Langman (1975), although BW would appear to be in the lower part of the normal range for the fetuses above 30 cm CR. The cubic power relationship between BW and CR means that at the end of the first half of gestation CR is about half that of the full term, but BW is only about one-seventh of the full term.

As far as the gross muscle parameters are concerned (Fig. 2), there are some discrepancies between these results and the results of MacCallum (1898). MacCallum found m. sartorius in five fetuses to be all slightly longer than those at similar CR shown here, e.g. at 20 cm CR he gave 5·2 cm as the length (contrast the 4·6 cm given here – Fig. 2*b*). The main discrepancies, however, are in the cross sectional areas which MacCallum gave as 5·8 mm^2 at 17 cm CR and 8·4 mm^2 at 20 cm CR, both much higher than values shown here (Fig. 2*c*) although, conversely, his values at 7·4, 10·2 and 13·0 cm CR were lower than those given here. It is difficult to explain these discrepancies as there is no indication in MacCallum's paper as to how and in what position the muscles were fixed and subsequently treated.

There appears to be little other data available on gross parameters of individual muscles in prenatal development except for muscle weights in 36 bovine fetuses (Stickland, 1978), weights of various muscles in 11 porcine fetuses (Swatland, 1973) and muscle weights, lengths and widths in 40 ovine fetuses (Joubert, 1956*b*). In general, the results of these studies do not disagree with the trends shown in Figure 2. In particular, the fact that muscle length increase is the major factor contributing to muscle mass increase is in agreement with Joubert (1956*b*). It should be noted, however, that both Joubert (1956*b*) and Swatland (1973) found that rates of prenatal growth varied in muscles from different anatomical locations. Hence, the results shown in Figure 2 for m. sartorius may not be the same, in absolute terms, as the trends seen in other human muscles.

Effects of fixation

Before proceeding to the next section it is worth considering the effect of fixation on the various parameters investigated. The effect of formalin fixation on the weight of prenatal porcine muscle has been investigated in this laboratory and an increase in weight of 10–15% after fixation (no further processing) has been shown to occur.

Body weight, however, only increases by up to about 5 %, presumably due to other tissues being affected less than muscle. Joubert (1956*b*) also found little change in ovine fetus weights after fixation at all stages of gestation.

The effect of fixation and subsequent processing (as used in this present investigation) on cellular parameters has been investigated in this laboratory by Wigmore (personal communication) who has shown that both myotube and myofibre diameters in Araldite sections of porcine muscle are about 63 % of their diameters in frozen sections in early gestational stages and, at later stages, processed myofibres are about 70 % of the frozen ones. It is interesting to note that diameters of muscle fibres fixed in glutaraldehyde and embedded in Epon were also found to be about 70 % of the fresh fibre diameter (Eisenberg & Mobley, 1975) as were fibres fixed in either glutaraldehyde or formalin and embedded in wax (Stickland, 1975). The relative proportion of 'intercellular' space does not appear to be significantly affected at any gestational stage by the processing used here.

The effects of tissue processing do not alter the significance of any of the relative trends in muscle development which are concluded from the results. Even the fact that cell diameters are affected to a greater extent in early gestation than in late does not significantly affect the relative trends, owing to the fact that the difference is only 7 %. In other words, although the ordinate in some of the figures would be shifted up or down if allowance is made for fixation effects, the general shape of the curves and relative changes would not be affected. It was therefore decided not to make any corrections for fixation and processing effects but to discuss the results actually obtained from the processed tissue. It is useful, however, to be aware of these processing effects in any discussion of absolute rather than relative values.

Number and size of cells

The results of this investigation have shown that, in prenatal human muscle development, there is initially a double population of muscle cells, with the proportions of myotubes to myofibres being high at 7 cm CR, but rapidly decreasing to zero at about 21 cm CR or 550 g BW (Fig. 3), although the total number of myotubes shows little significant change over most of this period (Fig. 4). This double population of cells in developing muscle was noted in early work on human muscle by both MacCallum (1898) and Hewer (1927). MacCallum stated that myotube disappearance was lost by 17 cm CR whereas Hewer believed this was achieved later, by the 26th week (i.e. 25 cm CR from Fig. 1). The results of this investigation suggest that 21 cm CR is the time, i.e. between these two estimates, but this is quite different again from the 16th week (14 cm CR) given by Cuajunco (1942), but in closer agreement with the 20th week (about 19 cm CR) noted by Webb (1972). It is important to realise that some of the variation in these results may be due to different muscles being used, as it is known that different muscles develop at varying rates at the cellular level (e.g. Joubert 1956*b*).

The significance of the two populations of muscle cells in early development was probably first realized by Wohlfart (1937) who put forward a biphasic theory of muscle development, calling the larger myotubes B-fibres and the smaller myofibres A-fibres. This biphasic development has been shown in muscles of many animals, including the pig (Ashmore, Addis & Doerr, 1973; Swatland & Cassens, 1973*b*), lamb (Ashmore, Robinson, Rattray & Doerr, 1972), ox (Stickland, 1978), rat (Kelly & Zacks, 1969) and chick (Kikuchi, 1971). It is interesting to note that myotube appearance is lost by about mid-gestation in all the agricultural animals

mentioned, and this also appears to be the case in this present investigation on human fetuses. Various histochemical studies (e.g. Ashmore *et al.* 1972; Ashmore, Addis & Doerr, 1973; Fenichel, 1963) have shown that the large myotubes or B-fibres which develop first are destined to become slow-twitch fibres of adult muscle, whereas the smaller A-fibres (the large majority of which do not go through a distinct myotube stage) are destined to become fast-twitch fibres, although it is known (e.g. Davies, 1972) that some of these latter fibre types will convert to slow-twitch fibres during further development. It should be pointed out, however, that, unless certain histochemical tests are carried out, this double population of cells with different destinies can only be appreciated in early development, i.e. before the large myotubes have transformed into myofibres. It should also be noted that recently Beermann, Cassens & Hausman (1978) showed that, in completely fast portions of muscles, the large myotubes do not show slow-twitch histochemical properties, even in early development.

It can be seen from Figure 3 that, as well as myotube appearance being lost at about 21 cm CR (or 550 g BW), the number of myofibres per unit area changes from an increasing trend to a marked decrease at this same time. This distinct change must be due partly to a slowing down in the decrease of intercellular space (Fig. 8) and, perhaps more significantly, to the commencement of a more rapid fibre hypertrophy phase (Fig. 6). The decrease in intercellular space was noted in human muscle development by Dickerson & Widdowson (1960) and Widdowson (1969), and the significance of the chemical changes associated with these decreases is discussed by the same authors.

As far as cell size is concerned, there appears to be very little significant hypertrophy until about 22 cm CR and, in fact, there is an initial decrease in average cell size (due to the initially large myotubes decreasing in size) (Fig. 6) in agreement with Thurley (1972). The fact that myofibre hypertrophy is greatest in later gestation has also been shown quite clearly in sheep (Joubert, 1956*b*), pig (Thurley, 1972) and ox (Stickland, 1978). The data on cell cross sectional area in human sartorius muscle given by MacCallum (1898) show marked differences from the measurements given here. MacCallum gives 59 μm^2 at 7·4 cm CR, 23 μm^2 at 13 cm CR and 56 μm^2 at 20 cm CR (contrast Fig. 6). Part of the discrepancy, especially for the larger values from the smaller fetuses, may be due to the fact that MacCallum estimated fibre area by dividing a unit area by the number of fibres in that unit area, i.e. no provision for intercellular space was apparently made. Cuajunco (1940) gave results of fibre diameter measurements in m. sartorius at various stages and these are in close agreement with the results here (Fig. 7).

The histograms of Figure 7 show that the distribution width of myofibre diameters is the same from 7·5 cm CR to 23·0 cm CR. The slight increase in mean myofibre diameter (Fig. 6) is due to the smaller fibres increasing in size. It is not until 27·0 cm CR that the largest fibres start to hypertrophy. This is analogous to the situation in neonatal rat muscle (Ontell & Dunn, 1978) which shows no change in distribution width for fibre diameters in the first week post partum although the average size of fibre does increase. Rat muscle is particularly immature at birth, with fibre number increasing quite markedly after birth (Rayne & Crawford, 1975), and therefore more comparable, perhaps, with later gestational stages in the human.

The results shown here (Fig. 4) indicate that the rate of myofibre number increase, as seen in a complete muscle cross section, starts to slow down at about 22 cm CR, i.e. when hypertrophy starts to become more significant, but does not stop even up

to the time near birth. This is in contrast to MacCallum's (1898) results, which indicated that the number of fibres stopped increasing at 17 cm CR when the number in a sartorius section was 128×10^3 and, although the number was 152×10^3 at 20 cm CR, the number was 117×10^3 at birth. These three values do, however, stress the degree of individual variation in human sartorius muscle, which is why the results of Montgomery (1962) are difficult to interpret. Montgomery found that there were 101×10^3 fibres in a sartorius section at birth and 134×10^3 at four months of age. He rightly concluded that, from his results, it was difficult to say when fibre number had stopped increasing.

From Figure 9, also, it can be seen that hyperplasia, as measured by the number of myofibres in a complete muscle section, does not completely stop before birth. The contribution of hyperplasia to muscle cross sectional area increase is, however, greatly reduced to only about 6% near birth, with a concomitant sharp increase in the contribution of hypertrophy over the period studied. The initial 'negative hypertrophy' between 7·5 and 10 cm CR is due to the initial decrease in average cell size (Fig. 6). At this time hyperplasia accounts for all muscle cross sectional area increase. The contribution of hypertrophy shows a rapid increase at about 22 cm CR such that, at about 23·5 cm CR, there is an equal contribution by hyperplasia and hypertrophy. Near birth, as already mentioned, nearly all the muscle area increase is due to hypertrophy, with no more than a 6% contribution from hyperplasia. Therefore, although one cannot say that this hyperplasia does not stop before birth, its importance to muscle cross sectional area growth is not very significant near birth and, although there may be some hyperplasia after birth, its effect postnatally will be even less significant.

It is important to realise that the hyperplasia so far discussed has been that inferred from fibre counts made in one cross section at the mid-length level in m. sartorius, although counts made near the proximal or distal end would have yielded similar results according to Montgomery (1962). This measured hyperplasia is really a measure of 'apparent hyperplasia' in that increases in fibre length can cause an apparent hyperplasia by fibres growing into the level of the section. This phenomenon was probably first appreciated by MacCallum (1898) and has since been reiterated by many authors. It is difficult, however, from one section of each muscle, to estimate when real hyperplasia has ceased. Serial sections could be employed and then studied to obtain an estimate but this method would be extremely laborious. A possible alternative is to estimate, from histograms such as Figure 7, when there are no more fibres in the smallest size category. This probably then indicates when no new myofibres are forming. This latter method has been used by several authors to estimate the cessation of real hyperplasia and has been estimated to be by about two thirds of the gestation period in sheep (Joubert, 1956*b*; Swatland & Cassens, 1973*a*), pig (Swatland, 1973) and ox (Stickland, 1978). It has been mentioned in this investigation that 18·5 cm CR is the first stage when no myofibres in the smallest size class are present. There was, however, a frequency of two in this smallest class at 21·5 cm CR but none in this class thereafter. It should be mentioned that some of these very small fibres may be the tapering ends of larger fibres and not newly formed fibres. However, the tapering ends of fibres in developing porcine muscle appear very short in teased single fibre preparations, such that the tapering portion is seldom longer than about 10 μm (Wigmore, personal communication). An approximate estimate, based on the histogram results, would be 18 to 22 cm CR as the probable end of real hyperplasia but to avoid overestimation, due to the

possibility of sectioned tapering fibre terminations, it is probably better to take the lower end of this range. This rough estimate therefore indicates that real hyperplasia has ceased by about mid-gestation, which means that the hyperplasia phase of Figure 9 can be divided into a real hyperplasia phase (A) and an apparent hyperplasia phase (B). This latter phase is, of course, a result of longitudinal hypertrophy with the 'hypertrophy' phase being fibre cross sectional area hypertrophy.

Number of nuclei

There is a significant decline in the number of nuclei per unit area from about 22 cm CR (600 g BW) onwards (Fig. 3). This decline appears to continue postnatally as Stickland *et al.* (1975) working on pigs and Montgomery, Dickerson & McCance (1964) working on the fowl found that concentration of nuclei was highest in animals with a low body weight.

The results on total numbers of nuclei show that the increase in nuclei numbers in both whole sections (Fig. 4) and whole muscles (Fig. 5), though less so in the latter, slows down in later gestation. Montgomery's (1962) results on sartorius muscle nuclei showed a steady increase both in sections and whole muscles. His results were, however, based on counts of subsarcolemmal nuclei only, whereas this study included counts on all nuclei. As the source of muscle nuclei is from satellite cells, which are probably undifferentiated myoblasts (Stromer *et al.* 1974), it is conceivable that subsarcolemmal nuclei could increase at a greater rate than total nuclei. This indicates, however, that in later gestation satellite cell nuclei are incorporated into myofibres at a greater rate than they are dividing. As all nuclei were counted in this investigation it is possible to compare directly the results here with the biochemical results of Widdowson, Crabb & Milner (1972), whose estimates of total DNA content in human gastrocnemius muscle showed a slower rate of increase in later gestation, comparable with Figure 5. Protein/DNA ratios also increased markedly in later gestation, thereby indicating a decline in DNA concentration which also agrees with the results of Figure 3.

All the results on the numbers of myofibres, myotubes and nuclei so far discussed (Figs. 3 to 5; Table 1) have not shown that there is a closer relationship of these cellular parameters with either CR or BW. There is some evidence, however (Widdowson *et al.* 1972) that some muscle parameters (including total DNA) are lower in small-for-dates fetuses as compared to normal. All the fetuses in this investigation were, however, in the normal range of BW for given CR values. It would be interesting to repeat this sort of quantitative work on fetuses from both normal and small-for-dates populations and thereby investigate which parameters, if any, relate better to BW and which to CR, the latter probably being more related to chronological age.

Number of myofibrils

The results of Figure 10 show that, at any given cross sectional area, myofibres have more myofibrils than myotubes, which is understandable by virtue of the fact that myofibres are uniformly filled with myofibrils whereas myotubes, by definition, contain only a peripheral ring of myofibrils.

It has been shown here that, during development, myotubes decrease in cross sectional area (Fig. 6). It would seem that this decrease in size is not associated with any change in the number of myofibrils (Fig. 10) during the period studied. Fidzińska (1971), however, observed an increase in the number of myofibrils in myo-

tubes in early human embryos between the 9th and 16th week, i.e. up to about 14 cm CR. Taken as a whole, the results available suggest that, although there must be some initial increase in myofibril number in myotubes, there is probably no significant change in the latter half of the myotube's life, remaining at about 35 myofibrils per myotube. It would seem that, when the myotube reaches about 30 μm^2 in cross sectional area, it loses its myotube appearance and becomes a myofibre, and it is not until this stage that the number of myofibrils start to significantly increase (Fig. 10). It is interesting to note that the relationship between myofibril number and cell size is not a linear one; myofibril number does not keep pace with size increase in later gestation. This type of curve is also seen postnatally in the mouse (Goldspink, 1970). In the present investigation a 50 % increase in cell size from 30 μm^2 to 45 μm^2 corresponds to a 50 % increase in the number of myofibrils. In later gestation, however, a 50 % increase in cell size from 60 μm^2 to 90 μm^2 corresponds to only a 20 % increase in myofibril number. This phenomenon may be partly due to the fact that myofibrils also increase in size during growth, although it is known that longitudinal splitting of myofibrils may occur when myofibril diameter is twice the normal, and this in fact seems to be the mechanism whereby myofibrils increase in number during growth (Goldspink, 1970; Shear, 1974).

CONCLUSIONS

It has not been the aim of this investigation to elaborate the mechanisms of prenatal muscle development, but to quantify the cellular changes which take place and so build up an accurate time-sequence of cellular events. The most important results of this investigation are probably those summarised in Figure 9 describing the rate of changeover from hyperplasia to hypertrophy during gestation.

As was stated in the Introduction to this investigation, muscle size is determined by muscle fibre size and number and intercellular 'space'. Although there may be some genetic control over maximum fibre size, this is a very variable parameter and, from the many studies already mentioned, it appears that fibre number is far more genetically determined. There is some evidence, from experimental work on animals, that malnutrition during pregnancy affects ultimate cell number in the tissues of the offspring (Robinson, 1969; Widdowson, 1971). Winick & Noble (1966) also found that cell number in various tissues was affected by malnutrition in rats from birth to weaning, which could recover when subsequently adequately fed. As rats are relatively immature at birth this may be analogous to prenatal malnutrition effects in other animals. From the results of this investigation it would seem that malnutrition *in utero* could affect muscle cell number (as estimated from a complete transverse section) in two ways. Firstly, malnutrition in the first half of gestation could affect real hyperplasia irreversibly. Secondly, malnutrition in the second half of gestation could reduce the apparent hyperplasia giving only an apparent affect of low cell number at birth. It is possible, however, that in this latter case adequate nutrition could allow recovery to take place by longitudinal hypertrophy of existing muscle fibres causing an increase in the fibre number apparent in a transverse section of muscle. As far as cell cross sectional area is concerned, malnutrition is likely to have a much more profound effect on this parameter in later gestation but, as with longitudinal hypertrophy, it is probable that this effect could be largely overcome by subsequent adequate nutrition.

The continuous change and rates of change in various cellular parameters shown

here have stressed the importance of describing muscle development in quantitative terms such as the graphical representations given here. It would be interesting to make a quantitative study of muscle development of, as already suggested, small-for-dates fetuses or of fetuses at risk for muscular dystrophy. These studies could then be compared with the present investigation and any significant differences more clearly defined in numerical terms.

SUMMARY

M. sartorius was removed from 21 human fetuses ranging from 7·0 to 35·0 cm crown–rump length (CR). Various gross and cellular changes (as seen in a transverse section) which take place in developing human skeletal muscle were quantified. The weights, lengths and cross sectional areas (at mid-length level) of m. sartorius were found to exhibit allometric relationships with CR and body weight (BW).

Initially (7·5 cm CR) myotubes were more numerous and larger (40 μm^2 cross sectional area) than the myofibres (about 26 μm^2). This situation was soon reversed, however, so that at about 19 cm CR myotubes were only a very small proportion of the total muscle cell population and somewhat smaller than the myofibres in cross sectional area. At about 21 cm CR all myotube appearance was lost, whilst the total number of myofibres increased rapidly up to about 22·5 cm CR, and thereafter the rate slowed down. This stage (22·5 cm CR) seemed, in fact, to be about the time when hypertrophy of myofibres started to markedly replace hyperplasia as the main factor contributing to total muscle cross sectional area increase, although there was still a 6 % contribution from hyperplasia at 35 cm CR. At 18 to 22 cm CR there were no more myofibres in the smallest size class (2–3 μm diameter). This may be an indication that real hyperplasia had ceased at this point so that beyond this the hyperplasia seen was only apparent and represented longitudinal growth of existing myofibres.

Throughout the period studied the amount of intercellular space decreased (at a declining rate) from about 62 % at 7 cm CR to about 21 % at 35 cm CR. Results on counts of nuclei suggested that total muscle nuclear proliferation slowed down in later gestation. Myofibril number was not related to myotube size but increased, though at a declining rate, with myofibre size. All the muscle parameters mentioned were plotted against CR, and sometimes BW, and regression equations given wherever possible.

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REFERENCES

ABERCROMBIE, M. (1946). Estimation of nuclear population from microtome sections. *Anatomical Record* **94**, 239–247.

ADAMS, R. D. (1975). *Diseases of Muscle. A Study in Pathology*. 3rd ed. New York: Harper & Row.

ASHMORE, C. R., ADDIS, P. B. & DOERR, L. (1973). Development of muscle fibers in the fetal pig. *Journal of Animal Science* **36**, 1088–1093.

ASHMORE, C. R., ROBINSON, D. W., RATTRAY, P. & DOERR, L. (1972). Biphasic development of muscle fibers in the fetal lamb. *Experimental Neurology* **37**, 241–255.

BEERMANN, D. H., CASSENS, R. G. & HAUSMAN, G. J. (1978). A second look at fiber type differentiation in porcine skeletal muscle. *Journal of Animal Science* **46**, 125–132.

CUAJUNCO, F. (1940). Development of the neuromuscular spindle in human fetuses. *Carnegie Institute, Washington Publication* 518. *Contributions to Embryology* **28**, 95–128.

CUAJUNCO, F. (1942). Development of the human motor end plate. *Carnegie Institute, Washington Publication* 541. *Contributions to Embryology* **30**, 127–152.

DAVIES, A. S. (1972). Postnatal changes in the histochemical fibre types of porcine skeletal muscle. *Journal of Anatomy* **113**, 213–240.

DICKERSON, J. W. T. & WIDDOWSON, E. M. (1960). Chemical changes in skeletal muscle during development. *Biochemical Journal* **74**, 247–257.

EISENBERG, B. R. & MOBLEY, B. A. (1975). Size changes in single muscle fibers during fixation and embedding. *Tissue and Cell* **7**, 383–387.

ELIOT, T. S., WIGGINTON, R. C. & CORBIN, K. B. (1943). The number and size of muscle fibers in the rat soleus in relation to age, sex and exercise. *Anatomical Record* **85**, 307–308.

FENICHEL, G. M. (1963). The B fiber of human fetal skeletal muscle. A study of fiber diameter size. *Neurology* **13**, 219–226.

FIDZIANSKA, A. (1971). Electron microscopic study of the development of human foetal muscle, motor end-plate and nerve. *Acta neuropathologica* **17**, 234–247.

GOLDSPINK, G. (1962). Studies on postembryonic growth and development of skeletal muscle. *Proceedings of the Royal Irish Academy* B **62**, 135–150.

GOLDSPINK, G. (1964). The combined effects of exercise and reduced food intake on skeletal muscle fibres. *Journal of Cellular and Comparative Physiology* **63**, 209–216.

GOLDSPINK, G. (1970). The proliferation of myofibrils during muscle fibre growth. *Journal of Cell Science* **6**, 593–604.

HAMMOND, J. & APPLETON, A. B. (1932). *Study of the Leg of Mutton. Part V. Growth and Development of Mutton Qualities in the Sheep.* London: Oliver & Boyd.

HEWER, E. E. (1927). The development of muscle in the human foetus. *Journal of Anatomy* **62**, 72–78.

HUXLEY, J. S. (1924). Constant differential growth ratios and their significance. *Nature* **114**, 895–896.

JOUBERT, D. M. (1955). Growth of muscle fibre in the foetal sheep. *Nature* **175**, 936–937.

JOUBERT, D. M. (1956*a*). An analysis of factors influencing post-natal growth and development of the muscle fibre. *Journal of Agricultural Science* **47**, 59–102.

JOUBERT, D. M. (1956*b*). A study of pre-natal growth and development in the sheep. *Journal of Agricultural Science* **47**, 382–428.

KARNOVSKY, M. J. (1965). A formaldehyde–glutaraldehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology* **27**, 137 A.

KELLY, A. M. & ZACKS, S. I. (1969). The histogenesis of rat intercostal muscle. *Journal of Cell Biology* **42**, 135–154.

KIKUCHI, T. (1971). Studies on the development and differentiation of muscle. III. Especially on the mode of increase in the number of cells. *Tohuku Journal of Agricultural Research* **22**, 1–15.

LANGMAN, J. (1975). *Medical Embryology*. 3rd ed. Baltimore: Williams & Wilkins.

MACCALLUM, J. B. (1898). On the histogenesis of the striated muscle fibre and the growth of the human sartorius muscle. *Johns Hopkins Hospital Bulletin* **9**, 208–215.

MEARA, P. J. (1947). Post-natal growth and development of muscle, as exemplified by the gastrocnemius and psoas muscles of the rabbit. *Onderstepoort Journal of Veterinary Science* **21**, 329–466.

MONTGOMERY, R. D. (1962). Growth of human striated muscle. *Nature* **195**, 194–195.

MONTGOMERY, R. D., DICKERSON, J. W. T. & MCCANCE, R. A. (1964). Severe undernutrition in growing and adult animals. 13. The morphology and chemistry of development and undernutrition in the sartorius muscle of the fowl. *British Journal of Nutrition* **18**, 587–593.

ONTELL, M. (1977). Neonatal muscle: an electron microscopic study. *Anatomical Record* **189**, 669–690.

ONTELL, M. & DUNN, R. F. (1978). Neonatal muscle: a quantitative study. *American Journal of Anatomy* **152**, 539–555.

RAGSDALE, A. C. (1934). Growth standards for dairy cattle. *University of Missouri: Agricultural Experiment Station, Research Bulletin* **336**, 1–12.

RAYNE, J. & CRAWFORD, G. N. C. (1975). Increase in fibre numbers of the rat pterygoid muscles during postnatal growth. *Journal of Anatomy* **119**, 347–357.

ROBINSON, D. W. (1969). The cellular response of porcine skeletal muscle to prenatal and neonatal nutritional stress. *Growth* **33**, 231–240.

ROWE, R. W. D. & GOLDSPINK, G. (1969). Muscle fibre growth in five different muscles in both sexes of mice. I. Normal mice. *Journal of Anatomy* **104**, 519–530.

SHEAR, C. R. (1974). Myofibril proliferation in developing skeletal muscles. In *Recent Advances in Myology* (ed. W. G. Bradley, D. Gardner-Medwin & J. N. Walton), pp. 364–373. Amsterdam: Elsevier.

SNEDECOR, G. W. & COCHRAN, W. G. (1967). *Statistical Methods.* 6th ed. Ames, Iowa: The Iowa State University Press.

SPIRO, A. J., SHY, G. M. & GONATAS, N. K. (1966). Myotubular myopathy: persistence of fetal muscle in an adolescent boy. *Archives of Neurology* **14**, 1–14.

STAUN, H. (1963). Various factors affecting number and size of muscle fibres in the pig. *Acta agriculturae scandinavica* **13**, 293–322.

STICKLAND, N. C. (1975). A detailed analysis of the effects of various fixatives on animal tissue with particular reference to muscle tissue. *Stain Technology* **50**, 255–264.

STICKLAND, N. C. (1978). A quantitative study of muscle development in the bovine foetus (*Bos indicus*). *Anatomia Histologia Embryologia* **7**, 193–205.

STICKLAND, N. C. & GOLDSPINK, G. (1973). A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16**, 135–146.

STICKLAND, N. C., WIDDOWSON, E. M. & GOLDSPINK, G. (1975). Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscle of pigs. *British Journal of Nutrition* **34**, 421–428.

STROMER, M. H., GOLL, D. E., YOUNG, R. B., ROBSON, R. M. & PARRISH Jr, F. C. (1974). Ultrastructural features of skeletal muscle differentiation and development. *Journal of Animal Science* **38**, 1111–1141.

SWATLAND, H. J. (1973). Muscle growth in the fetal and neonatal pig. *Journal of Animal Science* **37**, 536–545.

SWATLAND, H. J. & CASSENS, R. G. (1973*a*). Inhibition of muscle growth in foetal sheep. *Journal of Agricultural Science* **80**, 503–510.

SWATLAND, H. J. & CASSENS, R. G. (1973*b*). Prenatal development, histochemistry and innervation of porcine muscle. *Journal of Animal Science* **36**, 343–354.

THURLEY, D. C. (1972). Increase in diameter of muscle fibres in the foetal pig. *British Veterinary Journal* **128**, 355–358.

TOMANEK, R. J. & COLLING-SALTIN, A.-S. (1977). Cytological differentiation of human fetal skeletal muscle. *American Journal of Anatomy* **149**, 227–246.

WEBB, J. N. (1972). The development of human skeletal muscle with particular reference to muscle cell death. *Journal of Pathology* **106**, 221–228.

WIDDOWSON, E. M. (1969). Changes in the extracellular compartment of muscle and skin during normal and retarded development. *Nutritio et Dieta* **13**, 60–68.

WIDDOWSON, E. M. (1971). Intra-uterine growth retardation in the pig. I. Organ size and cellular development at birth and after growth to maturity. *Biology of the Neonate* **19**, 329–340.

WIDDOWSON, E. M., CRABB, D. E. & MILNER, R. D. G. (1972). Cellular development of some human organs before birth. *Archives of Disease in Childhood* **47**, 652–655.

WINICK, M. & NOBLE, A. (1966). Cellular responses in rats during malnutrition at various ages. *Journal of Nutrition* **89**, 300–306.

WOHLFART, G. (1937). Über das Vorkommen verschiedener Arten von Muskelfasern in der Skelettmuskulatur des Menschen und einiger Säugetiere. *Acta psychiatrica et neurologica Suppl.* **12**, 1–119.

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Anatomy
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Scanning Electron Microscopy of Prenatal Muscle Development in the Mouse

N.C. Stickland
Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH, U.K.

Summary. Scanning electron microscopy was used to study prenatal muscle development in mouse fetuses ranging from 12 days to 18 days gestation. Some transmission electron micrographs were also used for comparison. At 12 days no myofibres were evident although at 13 days long fibres surrounded by many mononucleated cells could be seen. At 14 days bundles of primary myofibres were observed with new myofibres forming in crevices between adjacent fibres. At 16 days the primary myofibres had separated but smaller secondary myofibres could be seen forming along their surfaces. The myofibres were very compact at 18 days and often appeared fused but this was due to the basal lamina ensheathing clusters of primary myofibres with their secondaries. The scanning electron micrographs appeared to illustrate the theories of muscle development which have arisen out of the various studies employing sectioning techniques.

Key words: Muscle – Growth and Development – Ultrastructure

Introduction

Early events of muscle development have been studied using tissue culture techniques and some of these studies have employed scanning electron microscopy (Shimada 1972). Later events of in vivo muscle development have been studied using sectioning techniques often involving either histochemistry (Wirsén and Larsson 1964 in mice; Kamieniecka 1968 in humans; Ommer 1971 in calves; Ashmore et al. 1972 in lambs; Ashmore et al. 1973 in pigs) or transmission electron microscopy (Kelly and Zacks 1969 in rats; Kikuchi 1972 in chicks; Tomanek and Colling-Saltin 1977 in humans; Platzer 1978 in mice; Russell and Oteruelo 1981 in calves). In vivo muscle development does not appear to have been studied, however, using scanning electron microscopy.

The studies mentioned above and others involving sectioning techniques have led to the biphasic theory of muscle development which states that myoblasts line up and fuse to form, initially, a population of primary myofibres

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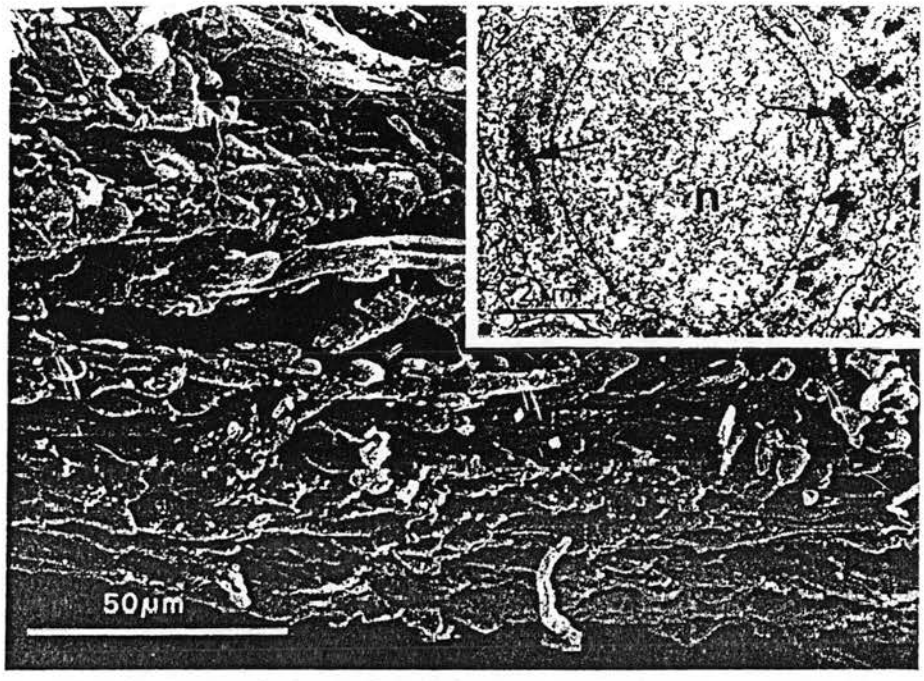

Fig. 1. Scanning electron micrograph of muscle from a 13 day fetus

Fig. 2. Transmission electron micrograph of a developing muscle fibre showing a central nucleus (*n*) and peripheral myofibrils (*arrows*)

(or myotubes as they are sometimes called owing to their appearance in section). Other myoblasts then line up on the surface of these to form a larger population of secondary myofibres.

This present investigation was carried out using the scanning electron microscope in order to try to illustrate this theory of muscle development and, in particular, to visualise the longitudinal arrangement of developing myofibres which may be difficult to appreciate in sections. Part of this work has been briefly reported in a previous communication (Stickland 1982).

Materials and Methods

Pregnant mice (C 57 strain) were obtained at daily intervals from 12 days gestation to 18 days. The fetuses were aged by using timed matings and confirmed by measuring crown-rump length and estimating the age by the data of Evans and Sack (1973). The full gestational period for this strain of mice is about 19 days. Whole forelimbs were removed from the fetuses and, after removing the skin, were placed in 3% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.3) for 1 h. For the larger fetuses (> 15 days gestation) m. biceps brachii was removed entirely and fixed for a further hour. For the smaller fetuses, however, the muscle was left in situ. The muscle was pulled apart to expose the developing muscle fibres and then, after rinsing in buffer, the samples were placed in 1% osmium tetroxide for 1 h. This was followed by dehydration in acetone before critical point drying in a Polaron critical point dryer. Freeze-drying techniques were tried

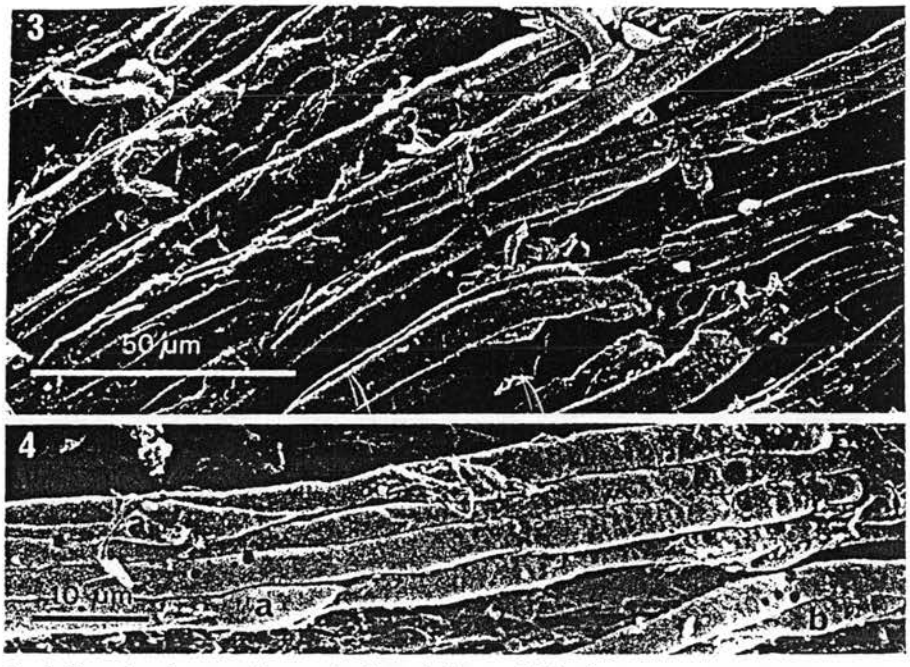

Fig. 3. Scanning electron micrograph of muscle from a 14 day fetus

Fig. 4. Higher power scanning electron micrograph of muscle from a 14 day fetus showing tapering ends (*a*) of small developing myofibres and possibly a single myoblast (*b*) in the crevices between adjacent myofibres of a bundle

but did not produce good results due possibly to the relatively large size of some of the samples. The muscles were viewed on an ISI-60 scanning electron microscope.

For comparison small muscle samples were also obtained for transmission electron microscopy. The samples were removed after the initial 1 h fixation in situ and then fixed for a further hour. The procedure was the same as above except that after dehydration the samples were embedded in Araldite. The sections were viewed on a Philips 400 transmission electron microscope.

Results

At 12 days gestation no fibres were seen in the region where m. biceps brachii would develop and in sections no myofibrils were observed in any of the cells. At 13 days, however, primary myofibres were clearly apparent (Fig. 1) although there were still many mononucleated cells present, representing undifferentiated cells, myoblasts, fibroblasts, etc. In section (Fig. 2) myofibrils could be seen in the developing muscle fibres. At 14 days, the myofibres were now very clear (Fig. 3) and there appeared to be fewer mononucleated cells present. The myofibres were arranged in bundes at this stage and a study of some of these bundles (Fig. 4) revealed the formation of smaller myofibres in the crevices between adjacent larger myofibres. Occasionally single mononucleated cells which could have been myoblasts were also seen in some of the crevices. At

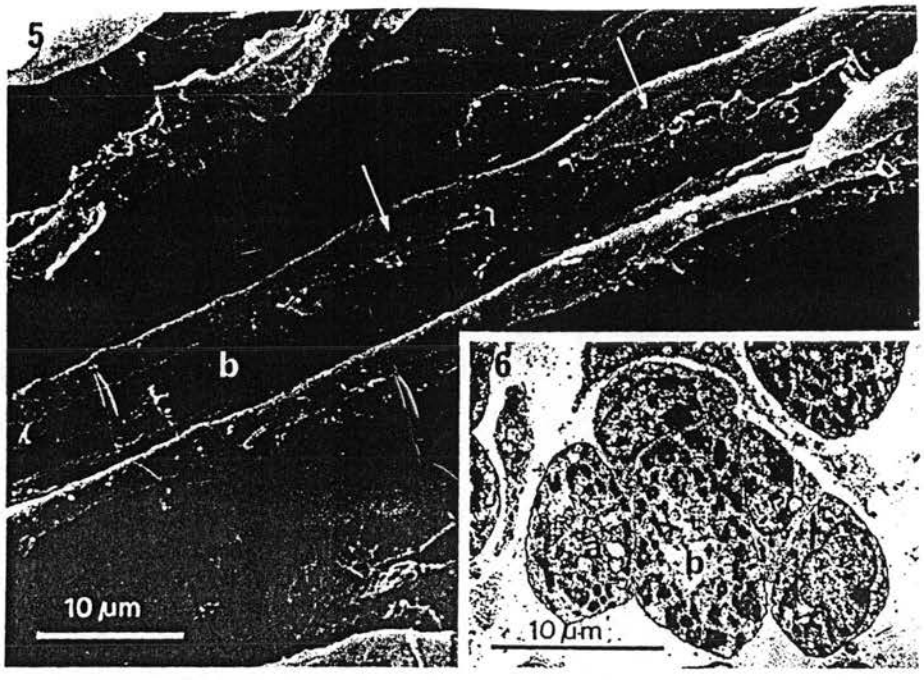

Fig. 5. Scanning electron micrograph of muscle from a 16 day fetus showing small secondary myofibres (*a*) on the surfaces of larger primary myofibres (*b*). The swellings (*arrows*) in the secondary myofibres probably denote the position of nuclei of fused myoblasts

Fig. 6. Transmission electron micrograph of muscle from a 16 day fetus showing a small secondary myofibre (*a*) adjacent to a larger primary myofibre (*b*)

16 days a range in diameter of myofibres was becoming evident. It could be seen on close examination (Fig. 5) that the primary myofibres were now no longer in bundles but that each large primary myofibre often had a smaller secondary myofibre closely applied to its surface. Collagen was starting to become more evident at this stage and can be seen in Fig. 5. In section (Fig. 6) clusters consisting of a large primary myofibre or myotube with one or two smaller secondary myofibres could be seen. At 18 days (Fig. 7) the fibres were much more compact although a range in diameters was still discernible. Fibre striations had become very clear by this stage indicating fairly close alignment of the myofibrils. Some fibres could be seen in very close contact so that they appeared to be almost fused. The sections (Fig. 8) at this stage showed the close contact between some adjacent fibres which were enclosed within a common basal lamina. This is presumably the reason for the apparent fusing seen with the scanning electron microscope.

Discussion

The absence of myofibres at 12 days is in agreement with Platzer (1978) who also found that many multinucleated myotubes were, however, present at

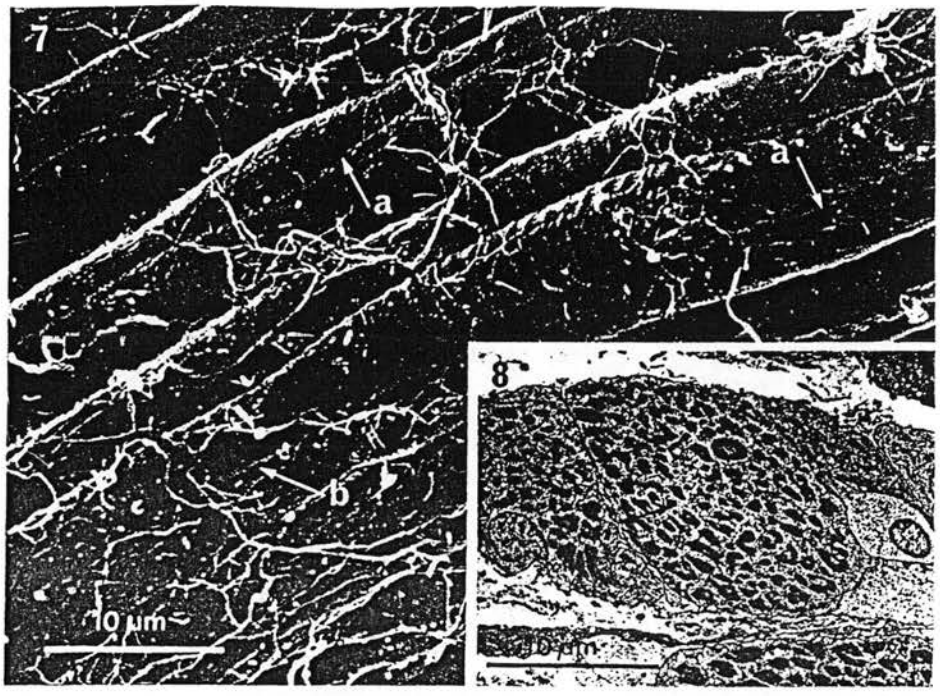

Fig. 7. Scanning electron micrograph of muscle from an 18 day fetus showing apparent fusion (*a*) between some adjacent myofibres although at some points (*b*) the 'fused' myofibres are clearly separated

Fig. 8. Transmission electron micrograph of muscle from an 18 day fetus showing the close contact (*arrows*) between some adjacent myofibres

14 days, although they may have been observed a little earlier (Fig. 1) in this present study, if it is assumed that the ages were accurately estimated.

In a study of muscle development in the rat, Kelly and Zacks (1969) described bundles of primary myotubes which then separated with each primary myotube forming its own 'cluster' with smaller secondary myofibres. In this present investigation the bundles of primary myotubes can be seen at 14 days (Fig. 3). New fibres forming at 14 days appeared to develop in crevices between existing fibres (Fig. 4). The initial population of fibres would seem to form a framework of crevices and surfaces for the formation of newer fibres.

The suggestion that myoblasts line up on the surface of primary myotubes to form secondary myofibres was put forward by Kikuchi (1971). This close association between primary and secondary fibres can be seen at 16 days in Fig. 5. The slight swellings in the secondary myofibres probably represent the nuclei of fused myoblasts.

The appearance at 18 days (Fig. 7) shows the apparent fusion between some adjacent fibres due to close contact between cells at this stage (Fig. 8) and to the basal lamina which surrounds each cluster of a primary fibre and its

secondaries (Kelly and Zacks 1969). The fact that this 'fusion' is often interrupted by regions where the fibres are clearly separated (Fig. 8) may be consistent with the findings of Ontell (1977) who, by a study of serial sections, showed that cells of a 'cluster' often left the ensheathing basal lamina.

Perhaps one of the most surprising observations made in this study was the relatively small number of fibre endings (Fig. 4) seen in any of the muscle samples viewed with the scanning electron microscope. Most authors who use sections to study muscle development suggest that much of the hyperplasia seen is not real hyperplasia but is due to fibres growing into the plane of section. Swatland and Cassens (1973), for example, suggest that over half the hyperplasia observed in sections of the sartorius muscle of foetal sheep is due to fibre elongation. It is highly unlikely that a contribution by fibre elongation to apparent hyperplasia can be as high as this in the mouse biceps brachii, as very few fibre endings were seen in this study. The situation in different muscles will probably vary, of course, but the scanning electron microscope could well be used to verify or otherwise the deductions made regarding the growth of intrafascicularly terminating fibres during development. Estimations of the proportions of fibres ending intrafascicularly at different ages could be made relatively easily.

Taken overall, this present investigation has demonstrated that the three-dimensional appearance of developing muscle is not inconsistent with the theories of muscle development. Scanning electron microscopy may be a useful technique for studying some aspects of in vivo muscle development especially, for example, for tracing developing fibres over some distance and for estimating the proportions of intrafascicularly terminating fibres in developing muscles for the reasons mentioned above.

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References

Ashmore CR, Robinson DW, Rattray P, Doerr L (1972) Biphasic development of muscle fibers in the fetal lamb. Exp Neurol 37:241–255

Ashmore CR, Addis PB, Doerr L (1973) Development of muscle fibres in the fetal pig. J Anim Sci 36:1088–1093

Evans HE, Sack WO (1973) Prenatal development of domestic and laboratory mammals. Growth curves, external features and selected references. Anat Histol Embryol 2:11–45

Kamieniecka Z (1968) The stages of development of human foetal muscles with reference to some muscular diseases. J Neurol Sci 7:319–329

Kelly AM, Zacks SI (1969) The histogenesis of rat intercostal muscle. J Cell Biol 42:135–153

Kikuchi T (1971) Studies on development and differentiation of muscle. III Especially on the mode of increase in the number of muscle cells. Tohoku J Agric Res 22:1–15

Ommer PA (1971) Histochemical differentiation of skeletal muscle fibres in the bovine foetus. Experientia 27:173–174

Ontell M (1977) Neonatal muscle: an electron microscopic study. Anat Rec 189:669–689

Platzer AC (1978) The ultrastructure of normal myogenesis in the limb of the mouse. Anat Rec 190:639–658

Russell RG, Oteruelo FT (1981) An ultrastructural study of the differentiation of skeletal muscle in the bovine fetus. Anat Embryol 162:403–417

Shimada Y (1972) Scanning electron microscopy of myogenesis in monolayer culture: a preliminary study. Dev Biol 29:227–233

Stickland NC (1982) A scanning electron microscopic study of prenatal muscle development in vivo. J Anat (Abstract) (in press)

Swatland HJ, Cassens RG (1973) Inhibition of muscle growth in foetal sheep. J Agric Sci 80:503–509

Tomanek RJ, Colling-Saltin A-S (1977) Cytological differentiation of human fetal skeletal muscle. Am J Anat 149:227–246

Wirsén C, Larsson KS (1964) Histochemical differentiation of skeletal muscle in foetal and newborn mice. J Embryol Exp Morphol 12:759–767

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Muscle development in large and small pig fetuses

P. M. C. WIGMORE* AND N. C. STICKLAND
Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH

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INTRODUCTION

The growth of muscle in agricultural animals is of interest because of its commercial importance. One of the aims of animal breeding and husbandry has been to produce animals with a greater mass of muscle. Under conditions of optimum feeding, postnatal muscle mass is related to muscle fibre number (Luff & Goldspink, 1967), a relationship seen in an extreme form in the enlarged muscles of double-muscled cattle (Ouhayoun & Beaumont, 1968; Swatland & Kieffer, 1974). Muscle fibre number is fixed before birth in the pig (Staun, 1972; Stickland & Goldspink, 1973) and so factors operating prenatally determine the maximum size to which this tissue can grow.

Widdowson (1971) has shown that the postnatal growth rate of low birth weight pigs is slower than that of their larger siblings, and that they fail to attain the same adult size even when fed *ad libitum*. It has also been reported (Widdowson, 1974; Powell & Aberle, 1981) that runt pigs have relatively smaller muscles than their larger littermates. Muscle fibre number was not directly counted in these studies, but both Hegarty & Allen (1978) and Powell & Aberle (1981) have concluded from indirect measurements that smaller pigs have fewer fibres at birth. Taken as a whole, these results suggest that prenatal factors which may affect total fibre number in a given muscle have a permanent effect on the postnatal growth of muscles in pigs.

The biphasic nature of fibre formation has been well established in the pig (Ashmore, Addis & Doerr, 1973; Swatland, 1973; Swatland & Cassens, 1973*a*; Beermann, Cassens & Hausman, 1978). According to this theory, an initial population of fibres, called primary fibres, is formed in the presumptive muscle by cell fusion. These fibres are then used as a surface for the attachment and fusion of myoblasts to form secondary fibres. Due to this mode of formation developing muscle fibres can be classified into two generations.

The purpose of the present investigation was to compare the prenatal development and growth of muscle in large and small porcine littermates. Particular attention was paid to total fibre number and to the possible mechanisms which might be responsible for a difference in this parameter between the littermates. A preliminary communication has already appeared (Wigmore & Stickland, 1981).

* Present address: Department of Anatomy, King's College, Strand, London WC2R 2LS.

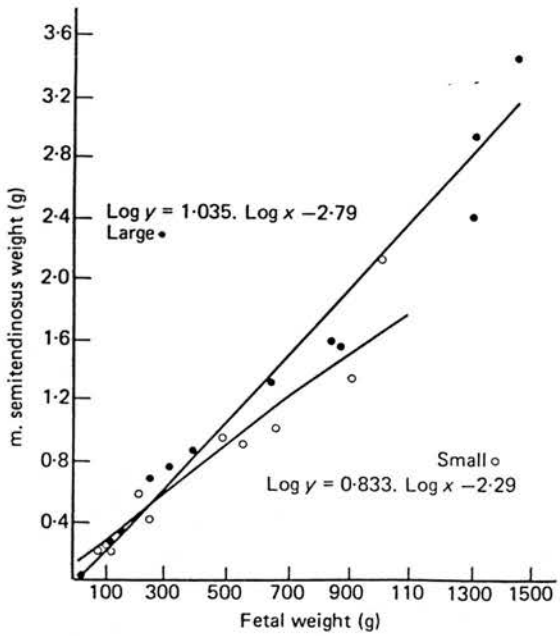


Fig. 1. The weight of the semitendinosus muscle from the largest and smallest littermates of each litter, plotted against fetal weight.

MATERIALS AND METHODS

All animals were obtained from a single farm and were gilts from a Large White/Landrace cross. Fetuses and neonatal animals were obtained from 38 days gestation until 1 day post partum, at intervals of approximately 7 days. The full gestational age of pigs is very constant at 114 days. At known gestational ages, pregnant gilts were slaughtered by standard abattoir procedures at a commercial abattoir, and the gravid uterus was removed. All fetuses in the litter were removed after ligation of the umbilical cord when the largest and smallest were chosen by weight. In three litters (102, 108 and 114 days' gestation) the lightest animal was identified as a runt by being more than two standard deviations below the mean weight of the rest of the litter. These animals appeared to constitute a separate population, and so the next lightest fetus was used as the small animal.

In all but the earliest ages, the left semitendinosus muscle was removed from each of the selected animals. A complete cross section was taken from the belly of this muscle and frozen in dichlorodifluoromethane (Arcton 12, I.C.I. Ltd.) cooled in liquid nitrogen. In animals of 38 and 46 days' gestation, it was impossible to dissect out the semitendinosus muscle and the whole limb was frozen. Sections of 10 μm thickness were cut on a Slee microtome at -25 °C and stained with haematoxylin and eosin.

Prenatal muscle fibres were classified as either primary or secondary (Ashmore, Robinson, Rattray & Doerr, 1972; Ashmore, Addis & Doerr, 1973; Swatland & Cassens, 1973*a*; Beermann, Cassens & Hausman, 1978). Primary fibres were recognised as those fibres with a tubular appearance due to either a central nucleus or a central myofibril-free region. Secondary fibres generally had a solid cross section and were found surrounding the primaries. Recognition was made easier

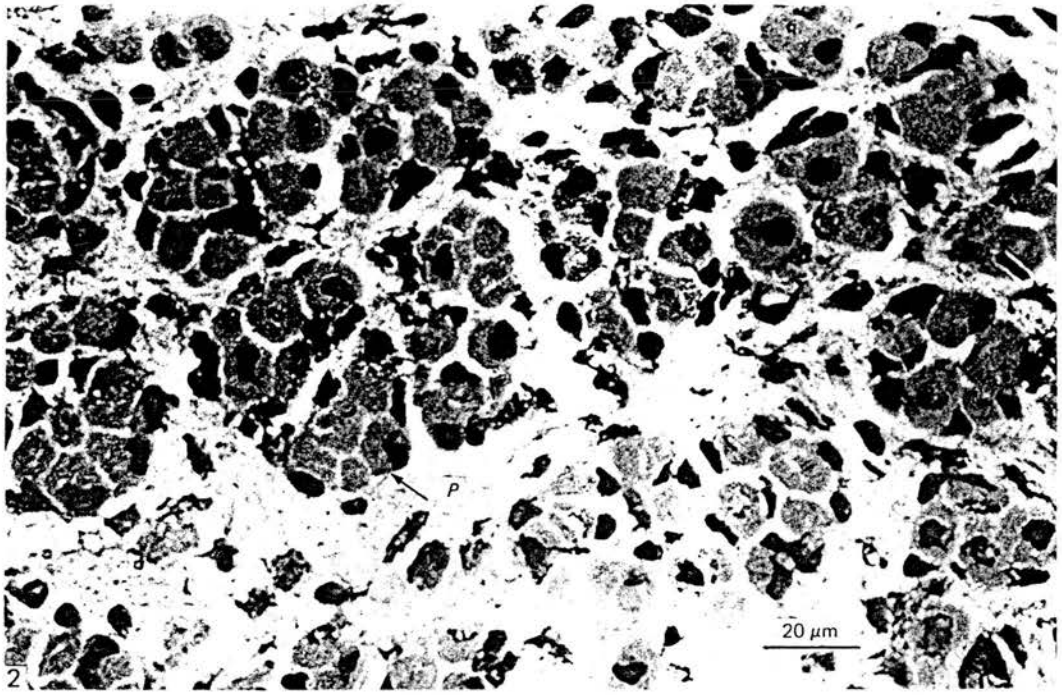


Fig. 2. A transverse section of the semitendinosus muscle from a fetus of 38 days' gestation. *P* indicates a primary fibre.

by the large size difference between these two generations of fibres, primary fibres being 2–3 times the size of the secondaries for most of the period studied. Sampling of each section was performed using 12 randomly chosen areas. Each area was photographed at magnifications of × 200 and × 400. The lower power pictures (area 0·136 sq. mm) were used to obtain the mean number of fibres per unit area which, together with the total section area, was used to estimate total fibre number. In the youngest animals (46 days' gestation) muscle cross sectional area was estimated by extrapolation from the results of older animals. The higher power pictures were used to obtain the mean fibre diameters for primary and secondary fibres. Approximately 120 fibres of each generation were measured from each section. The diameter measured was the maximum width of the fibre at right angles to its longest axis (Brook, 1970). This diameter was chosen in preference to fibre area because it is not subject to enlargement by olique sectioning.

Statistical analyses were carried out using the methods of Snedecor and Cochrane (1974).

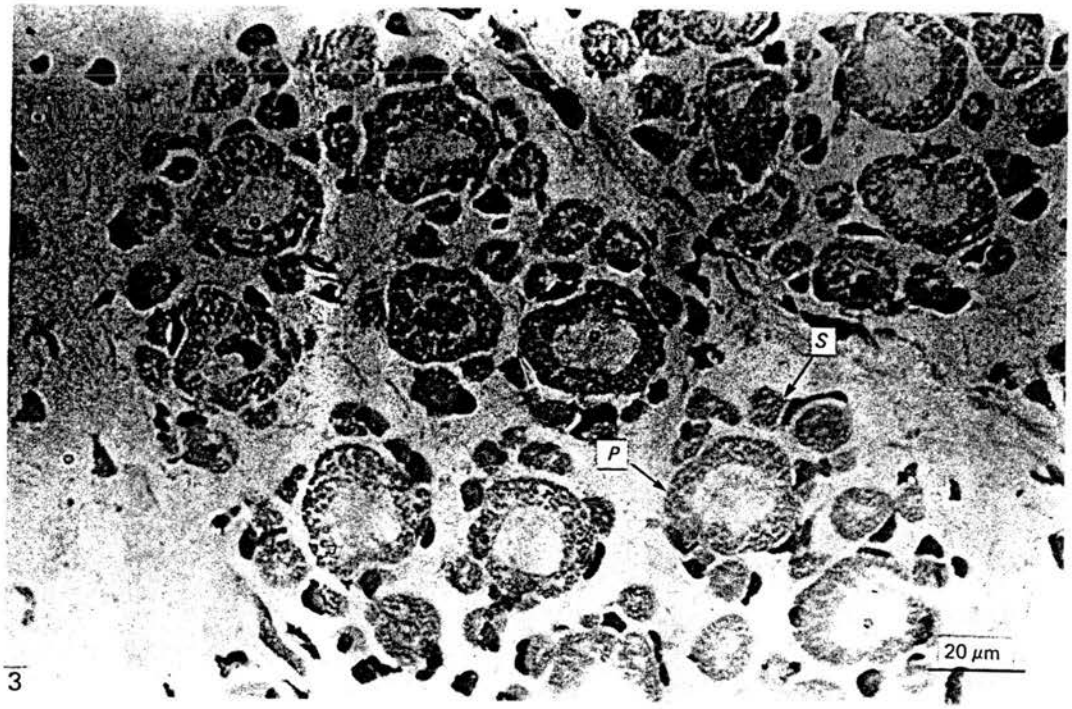
RESULTS

Muscle weight

The fresh weights of the semitendinosus muscles from the large and small littermates are shown plotted against fetal weight in Figure 1. The allometric equations for these two lines are shown on the Figure. The slopes of these lines are significantly different from one another ($P < 0{\cdot}05$), and that for the small fetuses is significantly less than 1·0 ($P < 0{\cdot}05$).

Histological appearance and fibre sizes

Sections of whole limbs at 38 days' gestation showed densely packed primary fibres in a broad band around the femur. These fibres had central nuclei and were



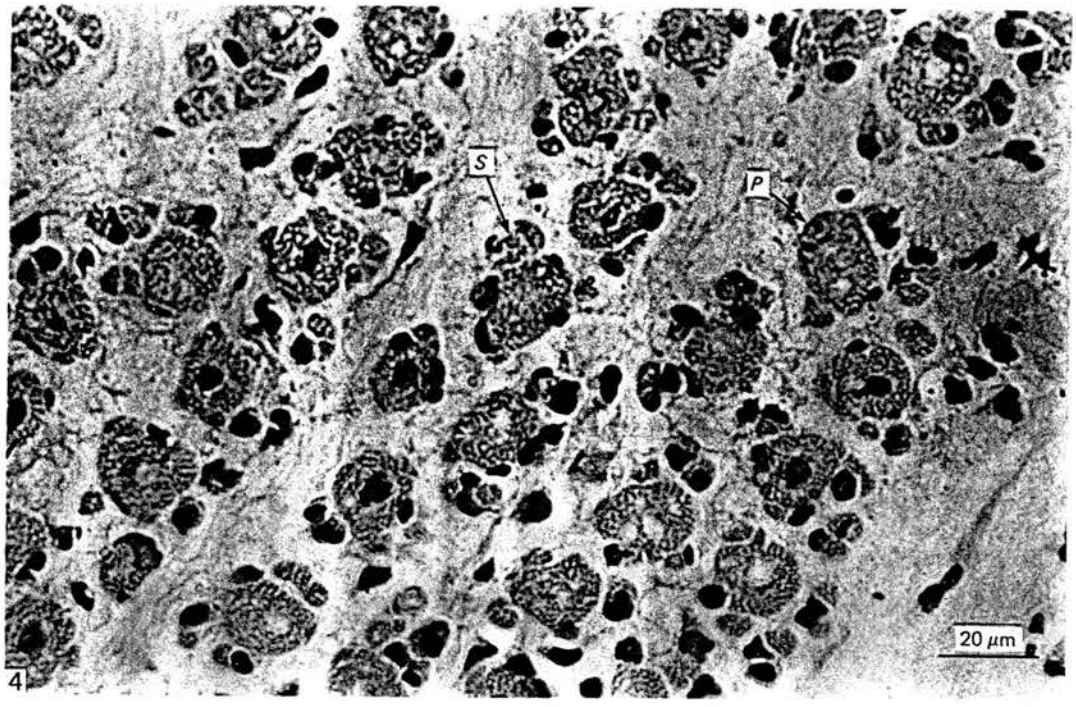


Fig. 3. A transverse section of the semitendinosus muscle of the largest fetus from a litter of 64 days' gestation. *P* indicates a primary fibre and *S* a secondary fibre.

Fig. 4. A transverse section of the semitendinosus muscle of the smallest fetus from a litter of 64 days' gestation. This animal came from the same litter as that shown in Fig. 3. *P* indicates a primary fibre and *S* a secondary fibre.

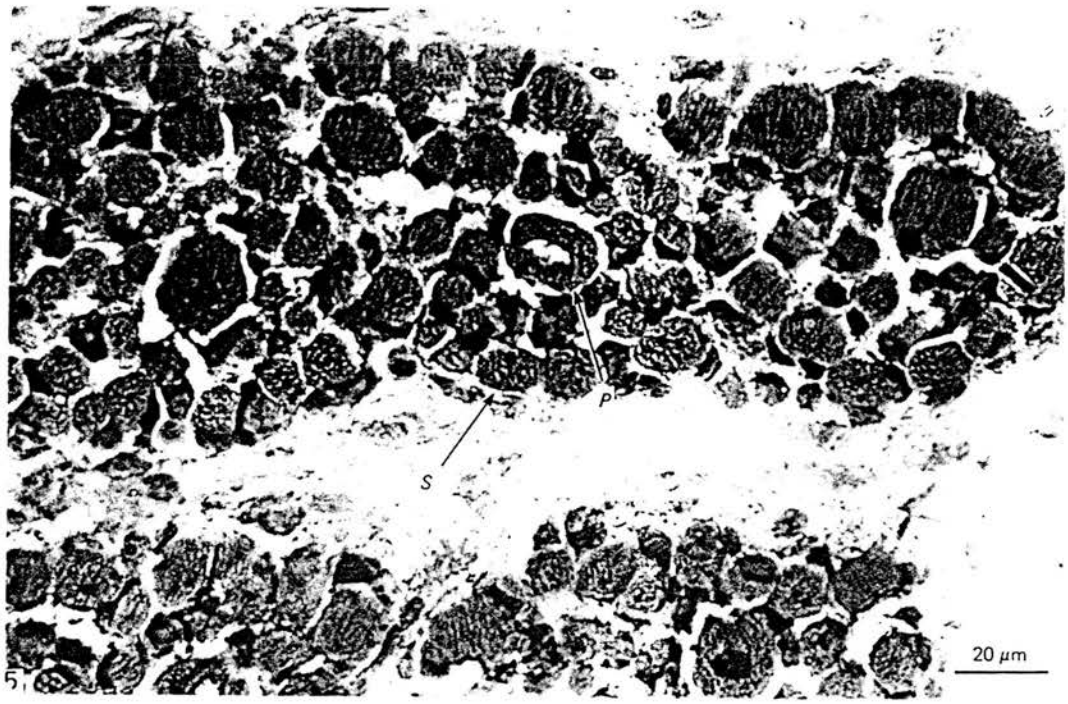


Fig. 5. A transverse section of the semitendinosus muscle from a fetus of 75 days' gestation. *P* indicates a primary fibre and *S* a secondary fibre.

surrounded by numerous mononuclear cells (Fig. 2). At this age, the fibres in both large and small fetuses were the same size (Fig. 6). By 46 days' gestation, connective tissue septa had begun to delineate individual muscles and the fibres in both large and small fetuses had increased in size (Fig. 6). Those in the large fetuses now had a greater mean diameter due to the formation of a region of myofibril-free cytoplasm in their centre. This feature did not appear in the fibres of small fetuses until 64 days' gestation (Fig. 4). Primary fibre size in both groups of animals continued to increase, reaching a maximum mean diameter of 22·8 μm at 64 days in the large littermates and 17·4 μm at 82 days in the small (Fig. 6). A paired *t*-test showed a significant difference ($P < 0{\cdot}05$) between large and small animals in primary fibre diameter during gestation. The major reason for this size difference was the greater development of the myofibril-free region in the fibres of large fetuses (Figs. 3, 4).

The first secondary fibres were seen at 54 days' gestation on the surfaces of the primary fibres. Their time of formation appeared to be the same in both large and small animals and, due to their continued formation, each primary fibre became surrounded by secondaries (Fig. 5). The smallest secondary fibres were seen on the surface of primary fibres but, after detaching from the fibres, their mean size remained relatively constant during gestation. A regression analysis showed that the slope of the line for secondary fibre diameter against age was not significantly different from zero. The mean diameter for secondary fibres was 7·8 μm in the large fetuses and 6·7 μm in the small fetuses. A paired *t*-test showed that throughout gestation there was a significant difference ($P < 0{\cdot}005$) in the secondary fibre diameter of large and small animals.

After 70 days' gestation, the size of primary fibres in both large and small litter-

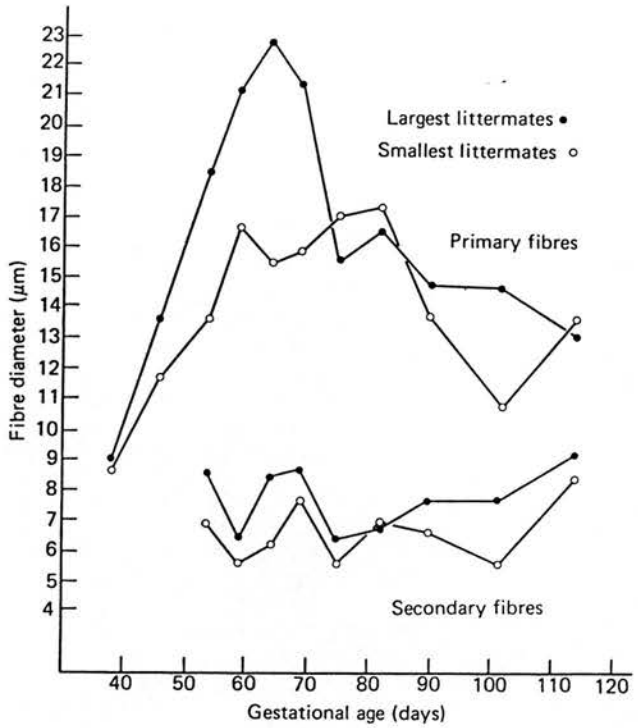


Fig. 6. Muscle fibre diameter, plotted against gestational age. Primary and secondary fibres for both the largest and smallest littermates are shown.

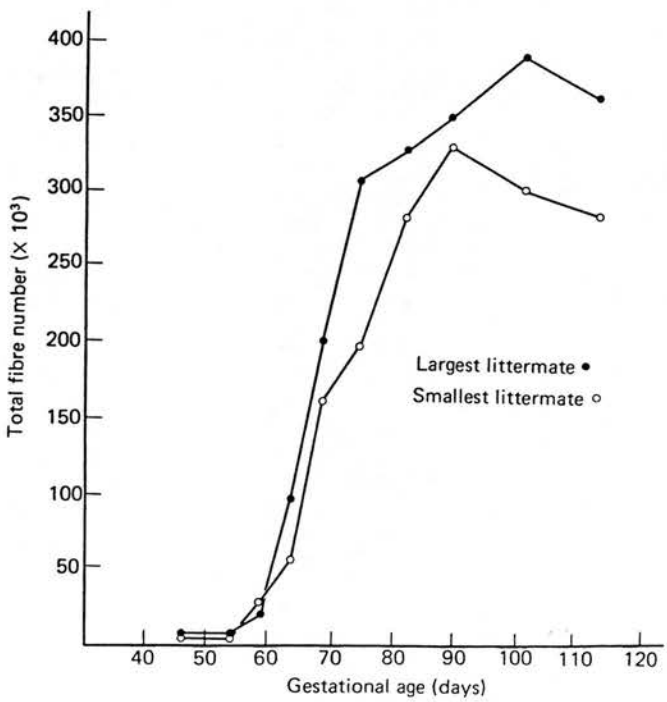


Fig. 7. Total fibre number in the semitendinosus muscle, plotted against gestational age for both the largest and smallest littermates.

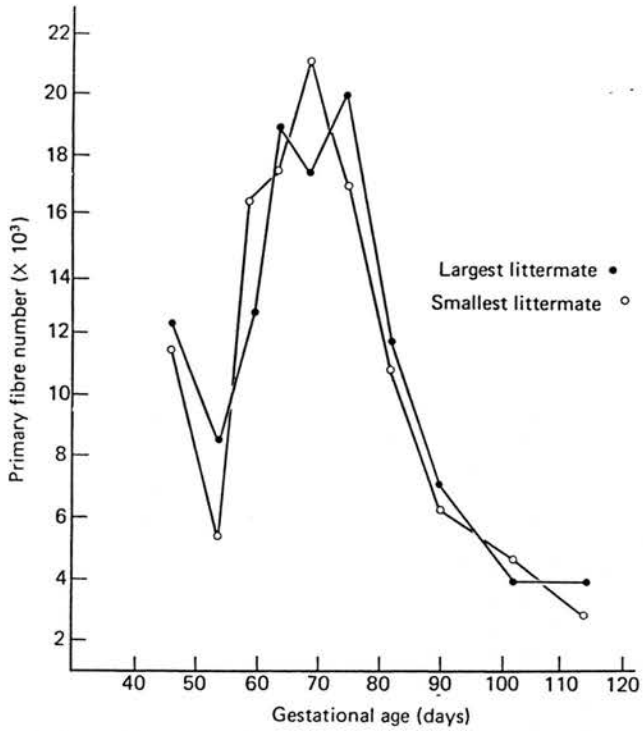


Fig. 8. The total primary fibre number in the semitendinosus muscle, plotted against gestational age for both the largest and smallest littermates.

mates declined as a result of the disappearance of the central myofibril-free region. This resulted in the primary fibres of both groups of animals becoming once again the same size. The decline in primary fibre size, and the subsarcolemmal migration of their nuclei, made it increasingly difficult to distinguish primary and secondary fibres in older animals. Therefore, the results after 80 days' gestation, shown in Figure 5, are those of recognisable primary fibres only. The adult appearance of the muscle was apparent by 100 days' gestation, when most fibres were of a uniform size, and connective tissue septa divided them into fascicles.

Fibre number

Figure 7 shows total fibre number in both large and small littermates. There was no difference in fibre number between the two groups of animals until after secondary fibre formation had started. After 64 days' gestation, the larger fetus always had more fibres at any given age, so that by birth there was a 17 % difference in total fibre number between large and small animals. Fibre formation appeared to cease between 85 and 90 days' gestation, by which time approximately 350000 fibres had formed.

Primary fibres reached their maximum number at about 60 days and then remained constant until 80 days (Fig. 8). The decline in primary fibre number after this age was due to the difficulty in distinguishing them from secondary fibres in older fetuses and not to the disappearance of primary fibres. It must be emphasised that the figures in this paper provide a quantitative description of the histological

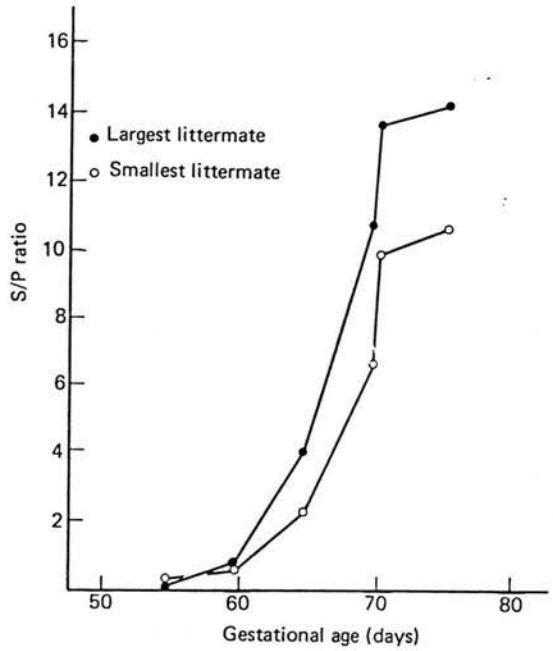


Fig. 9. The secondary/primary fibre ratio (*S/P* ratio), plotted against gestational age for both the largest and smallest littermates.

appearance of developing muscle. Approximately 20000 primary fibres were formed in the semitendinosus muscle. When primary fibre numbers in large and small fetuses were compared, by a paired *t*-test, during the period of secondary fibre formation (54–90 days), no significant difference in their numbers could be demonstrated. This would indicate that the fibre number difference observed at birth was due exclusively to fewer secondary fibres forming in the smaller littermates. This is illustrated in Figure 9 in which the average number of secondaries surrounding each primary fibre is given at various ages. The lines are not continued beyond 75 days due to the difficulty in distinguishing the two generations of fibres after this age. The final ratio can, however, be obtained from the total number of primary fibres formed (Fig. 8) and the total fibre number (Fig. 7). Using these methods, an average of 20 secondaries has been shown to form on the surface of each primary fibre in large fetuses compared to 16 in small fetuses.

DISCUSSION

The growth coefficient for the semitendinosus muscle of large littermates (Fig. 1) was not significantly different from 1·0, indicating that in these animals the semitendinosus muscle grows at the same rate as the whole body. The growth coefficient of this muscle relative to body weight was calculated, for postnatal well nourished Large White pigs, from the data of Davies (1973). Its value of 1·037 was not significantly different from that obtained prenatally for large littermates. This suggests that the optimum growth rate of the semitendinosus muscle is the same in the pre- and postnatal periods. The semitendinosus muscle of the small littermates grew significantly more slowly than body weight. This result is in agreement with Widdow-

son (1974) and Powell & Aberle (1981), who found that newborn low birth weight pigs had relatively smaller muscles than their larger littermates.

The cause of the size difference between large and small littermates is difficult to ascertain on an individual basis but, from gross measurements of body size (crown–rump length, ponderal index) and intrauterine position (unpublished observations), the small fetuses showed many signs of growth retardation consistent with prenatal malnutrition. This would explain the relatively lower growth rate of muscle in the small fetuses, because work on postnatal nutrition has shown that this tissue suffers a greater degree of growth retardation than the rest of the body (Dickerson & McCance, 1964; Widdowson, 1974). Malnutrition can also explain the fibre number difference between large and small animals because this has been produced under experimental conditions in a number of different species (Everitt, 1968: sheep; Swatland & Cassens, 1973*b*: sheep; Aziz-Ullah, 1974: mice; Bedi *et al.* 1982: rats).

The present work confirms the biphasic nature of muscle development in the pig. From 54 to 90 days' gestation there are clearly two populations of fibre which are distinguishable by their size, shape, and position within a fascicle. Factors affecting primary fibre formation are at present unknown. It is assumed that they form by fusion of myoblasts but, unlike secondary fibres, they do not appear to form on the surfaces of existing fibres. Secondary fibres were observed from 54 days onwards, and, due to their much higher rate of formation, came to constitute nearly 95 % of the final fibre population. The fibre number difference between littermates was due to a lower number of secondaries around each primary fibre in the small animals. This is likely to be permanent, because no change in fibre number occurs during postnatal growth (Staun, 1972; Stickland & Goldspink, 1973). The enlargement of primary fibres during secondary fibre formation may be to provide an increased surface area for the attachment and fusion of myoblasts forming secondary fibres. If this is so, then the smaller size of primary fibres in the small fetuses may be the cause of the lower numbers of secondary fibres forming on their surfaces. If this too is so, then factors affecting the development of the myofibril-free space within the primary fibres may be important in the control of fibre number.

It has been suggested by some authors (Swatland & Cassens, 1972) that differences in fibre number during growth can be explained by differences in the longitudinal growth of intrafascicularly terminating fibres. On the basis of this hypothesis, the fibre number differences found in the present work could be explained as resulting from the inhibition of the longitudinal growth of secondary fibres in small fetuses. However, teasing out of intact fibres and examination of longitudinal strips of muscle under the scanning electron microscope at several ages has failed to find any fibre ends from fibres of the size being counted (diameters greater than 2 μm), except at the ends of the muscle. The apparent absence of intrafascicularly terminating fibres makes it unlikely that intrafascicular growth can occur. Postnatally, low birth weight pigs fail to increase their muscle fibre number when fed adequately (Hegarty & Allen, 1978; Powell & Aberle, 1981). An increase in apparent fibre number might be expected if prenatal malnutrition had inhibited the longitudinal growth of intrafascicularly terminating fibres. While the possibility of changes in internal fibre architecture must be borne in mind in any growth study on muscle, the absence of any evidence for intrafascicular fibre growth in the present work leads the authors to believe that the fibre number differences reported here are real and are caused by differences in the rate at which secondary fibres form on the surface of primary fibres.

The finding in the present study that only secondary fibres are affected in the small fetuses suggests that the formation of these fibres is more susceptible to external influences than is that of the primaries. A similar result was reported by Harris (1981), who found that only primary fibres formed after chemically induced paralysis, or denervation. The effects of selection on domesticated animals are also thought to have increased the proportion of secondary fibres in their muscles (Ashmore *et al.* 1973).

In mixed fibre type muscles, fast twitch anaerobic fibres are formed exclusively from secondary fibres (Ashmore *et al.* 1973; Beermann *et al.* 1978). It is possible that a reduced number of secondaries will lead to a reduction in the proportion of this (fast twitch anaerobic) fibre type. It is interesting to note that this result has recently been found after experimentally induced malnutrition during pregnancy in rats (Bedi *et al.* 1982).

SUMMARY

The largest and smallest littermates were chosen by weight from litters of 38 days' gestation to 1 day post partum. Complete frozen sections of the semitendinosus muscle were used to provide a qualitative and quantitative account of the development of the primary and secondary generations of muscle fibres. The results showed that the time of formation of primary and secondary fibres, and the numbers of primary fibres formed, were the same in both large and small littermates. The number of secondary fibres formed, however, was lower in the smaller fetuses and resulted in there being a 17% difference in total fibre number at birth. Primary fibres in small fetuses were smaller, due to the smaller central myofibril-free region. This small size may have restricted the available surface area for secondary fibre formation. Fibre hyperplasia was found to cease between 85 and 95 days' gestation, and so the fibre number difference is likely to be permanent.

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REFERENCES

ASHMORE, C. R., ADDIS, P. B. & DOERR, L. (1973). Development of muscle fibres in the fetal pig. *Journal of Animal Science* **36**, 1088–1093.

ASHMORE, C. R., ROBINSON, D. W., RATTRAY, P. & DOERR, L. (1972). Biphasic development of muscle fibres in the fetal lamb. *Experimental Neurology* **37**, 241–255.

AZIZ-ULLAH (1974). Studies on muscle development with particular reference to the effects of protein malnutrition. Ph.D. thesis, Hull University.

BEDI, K. S., BIRZGALIS, A., MAHON, M., SMART, S. & WAREHAM, A. (1982). Early undernutrition in rats. I. Quantitative histology of skeletal muscles from underfed young and refed adult animals. *British Journal of Nutrition* **47**, 417–431.

BEERMANN, D. H., CASSENS, R. & HAUSMAN, G. (1978). A second look at fibre type differentiation in porcine skeletal muscle. *Journal of Animal Science* **46**, 125–132.

BROOK, M. H. (1970). Some comments on neural influence on the two histochemical types of muscle fibre. In *The Physiology and Biochemistry of Muscle as a Food*, vol. 2 (ed. E. J. Briskey, R. G. Cassens & B. B. Marsh), pp. 131–153. Madison: University of Wisconsin Press.

DAVIES, A. S. (1973). Postnatal development of porcine skeletal muscle. Ph.D. thesis, University of Edinburgh.

DICKERSON, J. W. & MCCANCE, R. A. (1964). The early effects of rehabilitation on the chemical structure of the organs and whole bodies of undernourished pigs and cockerels. *Clinical Science* **27**, 123–132.

EVERITT, G. C. (1968). Prenatal development of uniparous animals with particular reference to the influence of maternal nutrition in the sheep. In *Growth and Development of Mammals* (ed. G. A. Lodge & G. E. Lamming), pp. 131–157. London: Butterworths.

HARRIS, A. J. (1981). Embryonic growth and innervation of rat skeletal muscle. I. Neural regulation of muscle fibre numbers. *Philosophical Transactions of the Royal Society (Biol.)* **293**, 257–277.

HEGARTY, P. V. J. & ALLEN, C. (1978). Effect of pre-natal runting on the post-natal development of skeletal muscles in swine and rats. *Journal of Animal Science* **46**, 1634–1640.

LUFF, A. R. & GOLDSPINK, G. (1967). Large and small muscles. *Life Sciences* **6**, 1821–1826.

OUHAYOUN, J. & BEAUMONT, A. (1968). Étude du caractère culard. III. Anatomie microscopique comparée du tissu musculaire de mâles charolais normaux et culards. *Annales de Zootechnie* **17**, 213–223.

POWELL, S. E. & ABERLE, E. (1981). Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. *Journal of Animal Science* **52**, 748–756.

SNEDECOR, G. & COCHRANE, W. (1974). *Statistical Methods*, 6th ed. Iowa State University Press.

STAUN, H. (1972). The nutritional and genetic influence on number and size of muscle fibres and their response to carcass quality in pigs. *World Review of Animal Production* **8**/3.

STICKLAND, N. C. & GOLDSPINK, G. (1973). A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16**, 135–146.

SWATLAND, H. J. (1973). Muscle growth in the foetal and neonatal pig. *Journal of Animal Science* **37**, 536–545.

SWATLAND, H. J. & CASSENS, R. G. (1972). Muscle growth; the problem of muscle fibres with an intrafascicular termination. *Journal of Animal Science* **35**, 336–344.

SWATLAND, H. J. & CASSENS, R. G. (1973*a*). Prenatal development, histochemistry and innervation of porcine muscle. *Journal of Animal Science* **36**, 343–354.

SWATLAND, H. J. & CASSENS, R. G. (1973*b*). Inhibition of muscle growth in foetal sheep. *Journal of Agricultural Science* **80**, 503–509.

SWATLAND, H. J. & KIEFFER, N. M. (1974). Fetal development of the double muscled condition in cattle. *Journal of Animal Science* **38**, 752–757.

WIDDOWSON, E. M. (1971). Intra-uterine growth retardation in the pig. I. Organ size and cellular development at birth and after growth to maturity. *Biology of the Neonate* **19**, 329–340.

WIDDOWSON, E. M. (1974). Changes in pigs due to undernutrition before birth and for one, two and three years afterwards, and the effects of rehabilitation. *Advances in Experimental Medicine and Biology* **49**, 165–181.

WIGMORE, P. M. C. & STICKLAND, N. C. (1981). Prenatal muscle development in the pig; a comparison of the largest and smallest litter mates. *Journal of Anatomy* **133**, 132.

nat Embryol (1985) 173:263–268

Anatomy and Embryology

Placental growth in the pig

M.C. Wigmore and N.C. Stickland**
epartment of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH, U.K.

mmary. The growth of the porcine palcenta from 38 days ntil term is described. Measurements were made of its ea, circumference and in situ length. Placental area in-eased during the period of study due to increases in the erine circumference at the sites of conceptuses. No change as found in uterine horn length or placental length with e. Fetal weight correlated well with placental area but oorly with the other parameters. Placental length was orter in more crowded horns and showed a U shaped stribution with position within uterine horns. These re-lts are discussed in terms of competition for space within e uterus as a cause of the within litter variation in fetal ze.

ey words: Porcine placenta – Growth – Fetal size

troduction

here is considerable within and between litter variation the birth weights of pigs and a large part of this is thought be due to environmental effects acting within the uterus promote or depress the growth of individual animals. is likely that these effects are mediated through the pla-nta and work has been done on the within and between ter variation in placental growth shortly after implanta-on (Perry and Rowlands 1962; Webel and Dziuk 1974; nderson and Parker 1976; Anderson 1978). The present ork was undertaken to investigate placental growth from days until term. Of particular interest was the relation-ip between fetal and placental growth with reference to vironmental influences on placental growth that could plain variations in fetal size.

aterials and methods

l animals were obtained from the East of Scotland Col-ge of Agriculture farm at Easter Howgate and were gilts om a Large White, Landrace cross. The gilts were artifi-lly inseminated when they were between 100 and 120 kg e weight (approximately 165 days old) and the gestational es of the litters were calculated from the date this took place. At the required gestational age the gilts were slaughtered by standard abattoir procedures and the gravid uteri removed for dissection.

Thirteen gravid uteri were obtained at approximately 6 day intervals from 38 until 108 days gestation. The average gestational age at parturition for this herd was 114 days.

The fetuses were removed through a small incision in the uterine wall adjacent to each conceptus. The umbilical cord of each fetus was ligatured before being cut at its fetal end and both fetus and membranes were labelled. The fetuses were weighed and sexed from their external genitalia immediately after removal and then wrapped in clingwrap to prevent desiccation.

After removal of the fetuses, the antimesometrial border of the uterus was opened along its length. The length of the chorionic sac of each conceptus was measured in situ as well as the length of any unoccupied space between conceptuses. The distance from the ovary to the insertion of each umbilical cord on the placenta was measured to give the spacing of fetuses within the uterine horn.

After measurement in situ the fetal membranes were removed from the uterus, drained of their fluids and put into 10% formalin. After fixation each chorionic sac was cut so that it could be spread out over a flat surface to remove all macroscopic folds (Baur 1977), and a tracing made of its area. This was then measured on a Reichart Videoplan image analyser. The transverse circumference of the placental sac was obtained by measuring the maximum width of the opened uterus at the site of each fetus. Statistical methods were based upon Snedecor and Cochran (1967).

Results

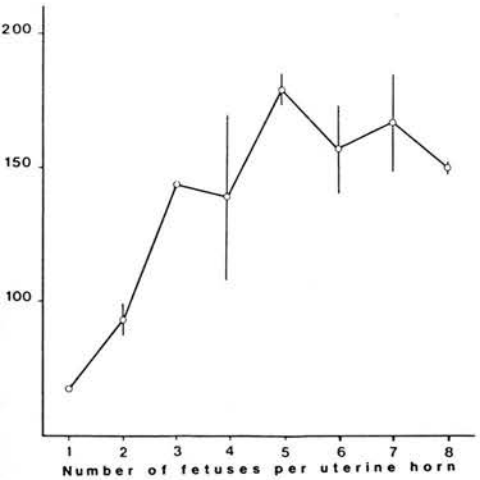
The mean number of fetuses per litter was 9.9 with a male to female ratio of 0.95:1.

Uterine measurements

The total length of each uterine horn was measured from the utero/fallopian tube junction to the cervix of twenty six horns carrying litters varying in age from 38 to 108 days from conception. The uterine horns showed no significant change in length with age (regression coefficient not significantly different from zero) either when all the data was pooled or when horns containing 4, 5, 6, or 7 fetuses were analysed separately.

rint requests to: P.M.C. Wigmore, Dept. of Anatomy, King's llege London, Strand, London WC2, U.K.

Present address: Dept. of Anatomy, Royal Veterinary College, Royal College St. London NW1, U.K.



g. 1. Mean length of horns plotted against number of fetuses esent. Bars represent plus or minus one standard error. Points thout bars are single observations

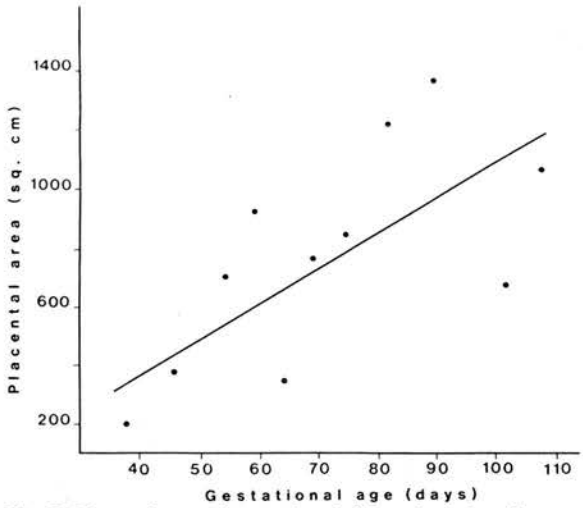


Fig. 2. Mean placental area of each litter plotted against gestational age. The regression coefficient of this line is significantly different from zero at the 1% level. The equation of the line is: $Y = 10.8 \times -8.5$

Differing numbers of fetuses also had little effect on rn length (Fig. 1). It is posible that the uterus shows increase in length when the number of fetuses increases om one to three but thereafter remains constant showing change up to the maximum number of eight fetuses r horn found in the present study.

acing of fetuses within the uterine horn

ıe spacing between fetuses within each uterine horn was termined by measuring the distance between the placental igins of the umbilical cords of adjacent fetuses whilst the acentae were still in situ. The results were analysed by e method of least squares for horns containing 4, (n=4, es 59, 64, 75, 108 days) 5, (n=2, ages 69, 82 days) 6, =ages 46, 70, 102 days) and 7, (n=4, ages 46, 69, 70, 2 days) fetuses. No significant differences could be dem-strated in the spacing of fetuses in different parts of the erine horn either with the raw data or when this was pressed as a percentage of the horn length.

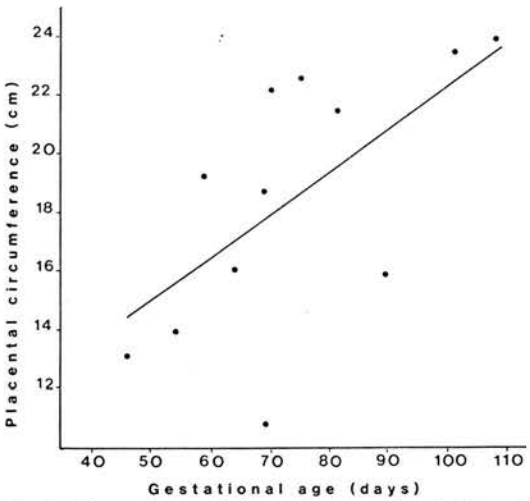


Fig. 3. Mean placental circumference of each litter plotted against gestational age. The regression coefficient of this line is significantly different from zero at the 1% level. The equation of the line is: $Y = 0.15 \times +7.9$

anges in placental parameters with age

gures 2 and 3 show placental area and circumference re-ectively plotted against gestational age. Equations are ven for the computed lines drawn on these figures in the ure legends. The regression coefficients for these lines e significant at the 5% level for placental circumference d the 1% level for placental area. No significant relation-p between placental length and age could be demon-ated either with the means for each litter or when horns ntaining the same number of fetuses were compared.

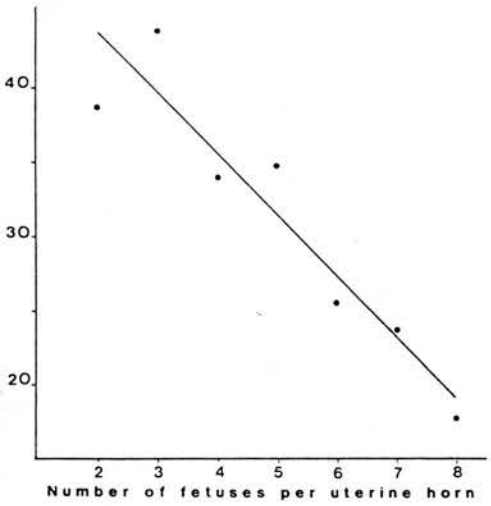
e effect of the number of fetuses per horn on the mean cental length and circumference

ıce placental length does not increase with age, the results m different ages may be plotted on the same graph. The ans of placental length from horns containing the same mber of fetuses were combined to produce Fig. 4. The equation of the computed regression line is given in the legend and the slope is significant at the 1% level. The graph shows a clear inverse relationship between the number of fetuses per horn and their mean placental length.

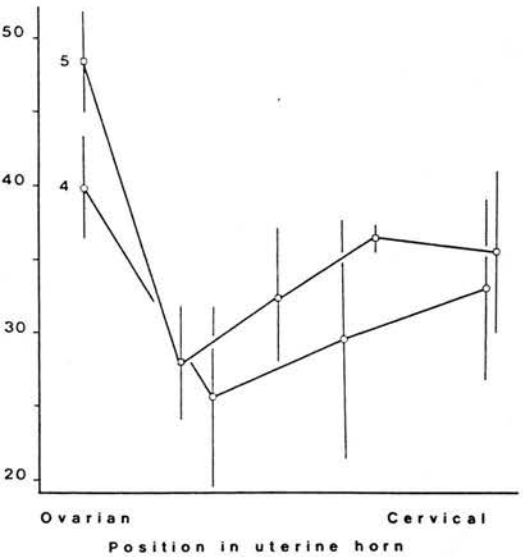
The effect of differing numbers of fetuses on placental circumference was analysed by comparing horns from the same uterus containing unequal numbers of fetuses in each horn. Eight pairs of horns were compared in this way using a paired *t*-test. Although the less crowded horns tended to have larger circumferences, no significant difference between the horns could be demonstrated.

The relationship between placental length and position within the uterine horn

The placental lengths from each horn were arranged in order from the most ovarian position to the most cervical.



. 4. Mean placental length within a horn plotted against the nber of fetuses present. The regression coefficient of this line ignificantly different from zero at the 1% level. The equation he line is: $Y = 51.5 - 4.0 \times$



. 5. The mean placental length at different positions within the rine horn for horns containing 4 or 5 fetuses (five horns con-ed 4 fetuses and three contained 5). Bars represent plus or min-one standard error. The position of each fetus is shown evenly ced from ovarian to cervical ends of the horn

e mean length for each position from horns containing same number of fetuses was then combined to produce s. 5 and 6. These graphs show the placentae spaced idistantly, as described above, for horns containing 4, 5 and 7 fetuses. In all cases the most ovarian placenta he longest and is significantly longer (more than two ndard deviations) than the placenta in the second posi-n for horns containing 5 or 6 fetuses. All four lines show shaped distribution where placentae in the most ovarian l cervical locations tend to be longer than those in more ermediate positions. This was tested by comparing the an of the most ovarian and cervical locations with the an of those in intermediate positions using a paired *t*-test

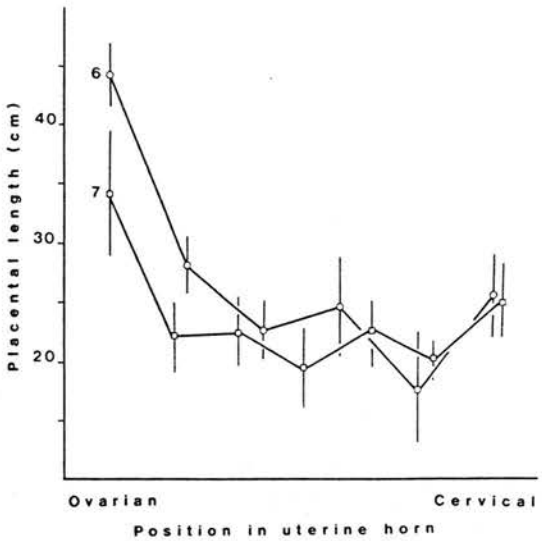


Fig. 6. The mean placental length at different positions within the uterine horn for horns containing 6 or 7 fetuses (four horns contained 6 fetuses and five contained 7). Bars represent plus or minus one standard error. The position of each fetus is shown evenly spaced from ovarian to cervical ends of the horn

Table 1. Correlation coefficients (*r*) between placental parameters and fetal weights for each litter. * Indicates a significant correlation at the 5% level. ** Indicates a significant correlation at the 1% level. NS indicates no significant correlation

Gestational age days	No of fetuses in litter	Placental area *r*	Placental length *r*	Placental circumference *r*
38	14	0.71**	0.54*	
46	13	0.82**	0.50 NS	0.15 NS
54	3	1.00**		0.93 NS
59	6	0.69 NS	0.40 NS	0.33 NS
59	11		0.64*	0.11 NS
64	13	0.41 NS	0.55 NS	0.57 NS
69	12	0.71**	0.62*	0.04 NS
70	13		0.67*	0.65*
75	7	0.82*	0.37 NS	0.27 NS
82	8	0.79*	0.37 NS	−0.10 NS
90	8	0.79*	0.28 NS	−0.14 NS
102	13	0.59*	0.47 NS	−0.74*
108	6	0.91*	0.65 NS	0.80 NS

for each horn within each group. The results showed significantly (5% level) longer placentae at the ends of horns carrying 6 or 7 fetuses but not for those carrying 4 or 5 fetuses. It is more difficult to analyse the present data for a position effect on fetal weight, placental area and placental circumference due to the increase in the values of these parameters with age. This was however attempted by expressing individual measurements as a percentage of the total value of these parameters for each horn. The means of these percentages then provided relative changes with position. Using this method no position effect could be demonstrated for any of the above parameters nor was there any significant difference between measurements from placentae or fetuses at the ends of the horns compared with those in more central positions.

Fig. 7. The inside lining of a pregnant uterus at the site of one conceptus. Scale bar = 2 cm

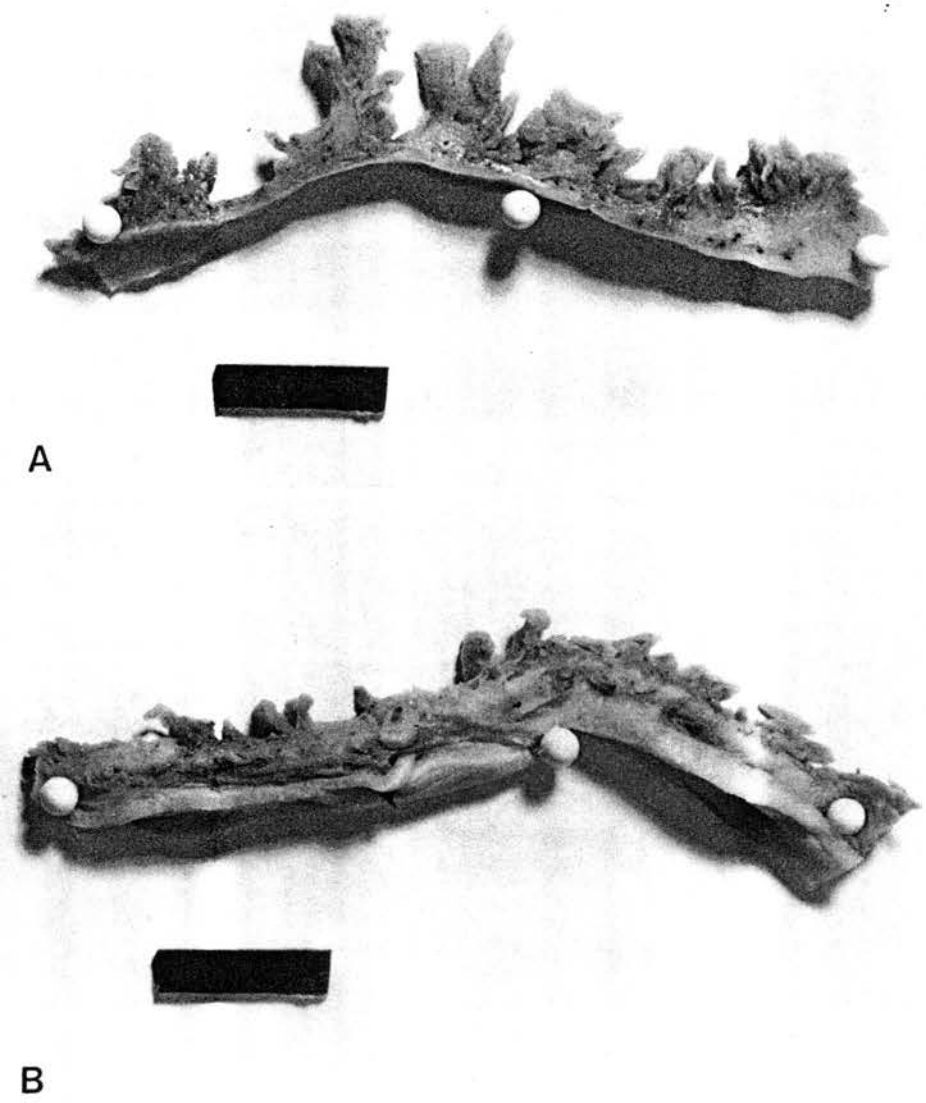


Fig. 8. Sections of the uterine wall from the implantation sites of two conceptuses from the same litter. Both sections are cut at right angles to the transverse folds shown in Fig. 6. The surface in contact with the fetal placenta is uppermost in both sections. Scale bar = 2 cm

e relationship between placental parameters and fetal ight

ble 1 shows the correlation coefficients between fetal ight and the area, length and circumference of placentae each litter. The most consistent and highest correlations those between fetal weight and placental area where but two litters showed a significant positive correlation. cental weight shows some correlation with fetal weight t placental length and circumference in general correlate orly with this parameter.

e increase in placental area by folds in the uterine lining

e above results which show that fetal weight does not rrelate well with either placental length or circumference re surprising, given the good correlation between fetal ght and placental areas. Examination of the inner sur- e of the pregnant uteri showed that, at the site of each nceptus, large transverse folds ran across the uterine rn. The outer surface of the placenta was found to con- m accurately to these folds before its removal from the rus. Figure 7 shows the internal lining of a pregnant rus at 64 days gestation. The area shown is that occupied a single conceptus and the maximum height of the folds etween 1 and 2 cms. The degree of folding varies consid- bly within a single uterus. Figure 8 shows sections of uterine wall from the implantation sites of two concep- es from the same litter. Each section has been cut from centre of the region occupied by the placenta and close he attachment of the mesometrial ligament. Both placen- from these regions had nearly the same area (A 1177sq ; B 1140sq cm) but that in A occupied a much shorter gth of uterus than B (25 cm compared to 46 cm). The ater height of the folds in the uterine lining in A is clearly dent.

cussion

cental area increases throughout gestation due to a con- nt increase in the circumference of the uterus and possi- by an increase in the degree of folding in the uterine ng. Placental area also shows a good correlation with l weight at all ages. The uterus however does not enlarge ccromodate increasing numbers of fetuses. The result hat larger litters have less space per animal. This is rly shown in Fig. 4 where there is a strong inverse rela- ship between the length of uterus occupied by each pla- ta and the number of fetuses present. Larger litters are refore more crowded and this competition for space is likely cause of the well known effect of small individual l weights occurring in large litters.

Variation in crowding between different parts of the e uterine horn cannot however explain variations in within a litter. For all numbers of fetuses the spacing eared to be even in all parts of the uterus. Measurements he distribution of implanted blastocysts (Anderson and ker 1976; Anderson 1978) also failed to detect any un- ness in spacing. Given that crowding can not explain in litter variations in fetal size it is possible that differ- es in placental size may be a cause in limiting fetal wth. Fetal size has been reported to vary in a character- way within the uterine horn. In many species (mice Mclaren and Mitchie 1960; rabbits Roshan and Greene 1936; Duncan 1969; guinea pigs Ibsen 1928; Garvis 1983) including the pig (Waldorf et al. 1957; Perry and Rowell 1969) the variation in fetal weight follows an approximately U shaped distribution down the uterine horn. The largest fetus is found at the most ovarian end of the horn and successively smaller fetuses are found towards the cervical region. The fetus located closest to the cervix however is larger than its more ovarian neighbour. This distribution was not found for fetal weight in the present study probably because of the rleatively small sample size and the variation caused by using litters of different ages. There appeared however to be a U shaped distribution of placental length within the uterine horns. In all groups (4, 5, 6 and 7 fetuses per horn) the longest placenta occurred at the most ovarian position and for horns containing 6 and 7 fetuses the placentae at the ends of the horns were significantly longer than those in more central locations.

Placental length is probably determined early in pregnancy when the blastocysts grow by extending a ribbon of tissue up and down the uterine horn (Perry and Rowlands 1962; Perry 1981). Placental length remains unchanged at least from 38 days gestation while other parameters of placental size increase with age. It is likely that fetuses at the ends of the uterine horns have longer placentae because they experience competition for space from only one side while placentae growing in intermediate positions have competition from both sides. In horns where a fetus has died the adjacent placentae expand into the vacated region of the horn. The longest placenta found in the present study (72 cm) occured at such a site. The placenta is not however entirely limited by the length of uterus originally occupied by the blastocyst. Folding of the uterine wall may greatly increase the surface area of the placenta and it is possible that those conceptuses occupying more restricted locations near the midle of the horn may still be able to develop placentae with relatively large surface areas.

Further work is needed to explain this variation in folding and also the factors affecting the determination of placental length early in pregnancy.

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References

Anderson LL (1978) Growth, protein content and distribution of early pig embryos. Anat Rec 190:143–154

Anderson LL, Parker RO (1976) Distribution and development of embryos in the pig. J Reprod Fertil 46:363–368

Baur R (1977) Morphometry of the placental exchange area. Adv Anat Embryol Cell Biol 53:3–65

Duncan S (1969) The partition of uterine blood flow in the pregnant rabbit. J Physiol (Lond) 204:421–433

Garvis DR (1983) Regional variations in guinea pig uterine blood flow during pregnancy. Relationships to intrauterine growth of the fetal-placental unit. Teratology 27:101–107

Ibsen HL (1928) Prenatal growth in guinea pigs with special reference to environmental factors affecting weight at birth. J Exp Zool 51:51–91

Mclaren A, Michie D (1960) Control of prenatal growth in mammals. Nature 187:363–365

Perry JS (1981) The mammalian fetal membranes. J Reprod Fertil 62:321–335

y JS, Rowell JG (1969) Variations in foetal weight and vascula-
ure supply along the uterine horn of the pig. J Reprod Fertil
9:527–534
y JS, Rowlands IW (1962) Early pregnancy in the pig. J Reprod
Fertil 4:175–188
ahn PD, Greene HS (1936) The influence of intrauterine factors
n the fetal weight of rabbits. J Exp Med 63:901–921
Lecor GW, Cochran WG (1967) Statistical methods. Sixth ed.
The Iowa State University Press, Ames Iowa
Waldorf DF, Foote WC, Self HL, Chapman AB, Cassida LE (1957) Factors affecting fetal pig weight late in gestation. J Anim Sci 16:976–985
Webel SK, Dziuk PJ (1974) Effect of stage of gestation and uterine space on prenatal survival in the pig. J Anim Sci 38:960–963

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The biceps brachii and soleus muscles (fast and slow twitch muscles, respectively) were taken from guinea-pig fetuses, undernourished via maternal dietary intake to 60% of ad lib, and examined for differences from their controls. At birth, muscle fiber number was found to be reduced by 26% in the biceps brachii but was unaltered in the soleus muscle. An examination of the muscles at ages through gestation revealed that any reduction in fiber number was associated with a reduction in the secondary/primary fiber ratio. There was a stage in soleus myogenesis at which fiber number was significantly lower than the control. The deficit was corrected by the continuation of secondary fiber hyperplasia beyond the time of usual cessation. A similar delay was found in the biceps brachii but it did not enable recovery to a full complement of fibers. The difference between the 2 muscles was thought to be related to the relative proportions of primary fibers in the muscles, primary fiber development being apparently unaffected by undernutrition in utero.
Key words: myogenesis • undernutrition • myofibers • guinea-pig
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WHY ARE SLOW AND FAST MUSCLES DIFFERENTIALLY AFFECTED DURING PRENATAL UNDERNUTRITION?

STEPHANIE S. WARD, BSc, PhD, and NEIL C. STICKLAND, BSc, PhD

Prenatal undernutrition may cause a reduction in muscle fiber number at birth and weaning.[2,13,14,26] Nutritional stress imposed after birth has no such effect on the number of muscle fibers.[25] Fiber hyperplasia ceases before birth in animals born at a relatively mature stage of development (pigs[30] and guinea-pigs[28]) and soon after birth in others (rat[2]). Undernutrition is, therefore, acting to reduce fiber number by limiting the number of muscle fibers formed during myogenesis.

Myogenesis is a biphasic process. An initial wave of myoblast fusion gives rise to a population of multinucleated primary fibers. Further myoblasts line up on the surface of these and fuse to form secondary fibers.[16,17,22] In the majority of developing muscles there are many more secondary fibers than primary fibers; however, the secondary/primary fiber ratio is lower in muscles or parts of muscles in which slow oxidative fibers predominate in the adult.[15]

In the absence of functional innervation, primary fibers will form, whereas, secondary fibers will not form or will regress.[12,19,24] The differential susceptibility of the 2 populations to nervous disruption seems to be extended to conditions of nutritional inadequacy.[28,30,31]

Muscles with adult slow twitch characteristics, such as the soleus muscle, invariably suffer less reduction in fiber number than other muscles when undernourished.[1,2,13,14,17] Since secondary fibers contribute less to the total number in presumptive slow twitch muscles, this may explain the comparative "sparing" of these muscles to undernutrition in utero. The present investigation was carried out in order to test the hypothesis that presumptive slow twitch muscles are less affected by undernutrition in utero than presumptive fast twitch muscles because the former contain a lower proportion of secondary fibers than the latter.

MATERIALS AND METHODS

Animals. Adult guinea-pigs of the Dunkin–Hartley strain of similar weights, ages, and breeding statuses were postpartum mated. Half the total number (total $n = 46$) were used as controls and to calculate a 60% of ad lib diet (SG1 pellets, Short

From the Department of Veterinary Basic Sciences, The Royal Veterinary College, London, England.

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Address reprint requests to N.C. Stickland, Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 OTU, England.

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nd Gammage) for the remaining guinea-pigs. The experimental animals were placed on the restricted diet immediately after conception. Both nutritional groups received a daily supplement of water miscible vitamin E (Roche) at a concentration of 60 mg of α tocopherol per kilogram of diet. The diet was found to satisfy all other vitamin requirements including vitamin C.

Animals were killed (with an intraperitoneal injection of sodium pentobarbitol) at 5-day intervals from 30 days to 55 days of gestation and then at term (average 68 days). There were normally 3 litters per gestational age for each nutritional group. All fetuses were removed and various gross body measurements were taken including body and organ weights (liver, brain, cerebellum, m. semitendinosus, m. soleus, m. biceps brachii) and crown-rump and bone lengths (tibia and humerus).

Muscle Selection. The biceps brachii and soleus muscles were selected for detailed analysis for 2 main reasons. First, the adult biceps brachii in the guinea-pig is mainly a fast twitch muscle. Slow oxidative (SO) fibers (as defined by Peter et al.[23]) comprise a mere 6% of the total number. In contrast, the adult soleus muscle is characterized by 100% SO fibers and is, therefore, a good example of a slow twitch muscle. Secondly, both muscles exhibit architectural simplicity. In the guinea-pig, although m. biceps brachii is a bipennate muscle, all the fibers are contained in the middle third of the muscle and the fibers run parallel to the long axis. The guinea-pig soleus is slightly unipennate but contains all its fibers running parallel to the long axis for most of its length. This means (and has been confirmed with acetyl-cholinesterase staining) that the motor end-plates cross the mid-belly of these muscles and all fibers can be located in a plane of sectioning taken from that area. This is particularly important in developing muscle where fibers grow outward from the motor end-plates. In terms of accurate fiber number counts the biceps brachii and soleus muscles were, therefore, ideal.

Histochemistry. The left biceps brachii and soleus muscles were mounted on cork and frozen in Arcton 12 (ICI Ltd.), cooled to −158°C in liquid nitrogen. Ten-micrometer sections were cut from the mid-belly of the muscle on a cryostat at a temperature of −20°C. Sections were reacted for adenosine triphosphatase in a basic medium (pH 10.6).[8] The resultant slides were used to: (1) estimate secondary/primary fiber (S/P) ratios and (2) monitor the changes in the histochemical staining pattern during myogenesis.

1. Secondary/Primary Fiber (S/P) Ratio. The basic ATPase reaction (BATPase) was used in order to be sure of estimating S/P ratio from actual muscle fibers. In the early stages of myogenesis there are many mononuclear cells such as myoblasts and fibroblasts whose only distinction from muscle fibers in cross section is the absence of myofibrils. BATPase does not react unless myofibrils are present. Nonmuscle fibers were, therefore, excluded from the calculations. Primary and secondary fibers were distinguishable up to and including 45 days of gestation in the control biceps brachii and soleus muscles and to 50 days in the undernourished muscles. A combination of criteria which changed with gestational age were used to identify them. These are outlined in the Results section. S/P ratio estimations were made for each muscle by counting all the primary fibers and their associated secondary fibers in ten complete fields across the whole muscle at a magnification of ×20.

Primary and secondary fibers initially form clusters in the same basement membrane.[21] It is possible, therefore, that S/P ratios could be underestimated with the light microscope because it might not be possible to distinguish small fibers within the cluster. Electron micrographs (Fig. 1) at a magnification of 2K were taken from a random selection of muscles at 40 days of gestation (which was a time of maximum secondary fiber formation) and used to investigate this possibility. No significant increase in S/P ratio was found when estimated from electron micrographs. This, therefore, justified the use of the light microscope for these estimations, especially as it would not be feasible to use the electron microscope for the large numbers of observations involved.

2. Histochemical Transitions. During development there are changes in the reaction of fibers to BATPase activity. While these changes cannot characterize myosin isozyme conversions in detail (for which myosin antibodies would be required) they do give some information on the timing of those changes.

Muscle Fiber Number Counts. The contralateral biceps brachii and soleus muscles were fixed in situ with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer and then processed for embedding in araldite resin (TAAB Laboratories Ltd.).[10] Whole 1 μm transverse sections were taken from

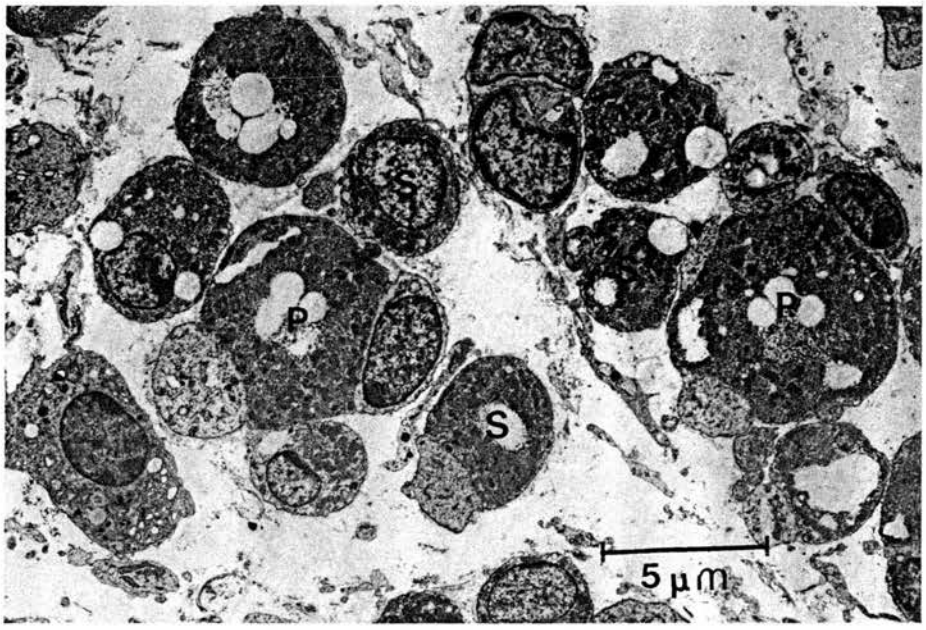


FIGURE 1. Control biceps brachii at 40 days of gestation. Electron micrograph. P = primary fiber; S = secondary fiber.

e mid-belly of the muscle and stained with 1% ethylene blue in 1% borax. Total muscle fiber unts were made from these sections using a pro-ction microscope.

Muscle fiber number counts were taken from sin embedded rather than frozen sections for 2 ain reasons. First, fixation in situ enabled sec-ns to be more confidently taken from the exact ddle of the muscle. With fresh, unfixed muscle, ntraction occurs when the muscle is removed d this makes it difficult to identify the midpoint the muscle. Secondly, it is easier to obtain a mplete transverse section of the whole muscle if is resin embedded rather than frozen. Parame-rs from the 2 experimental groups were com-red using an unpaired *t*-test except where an-ner test is specified.

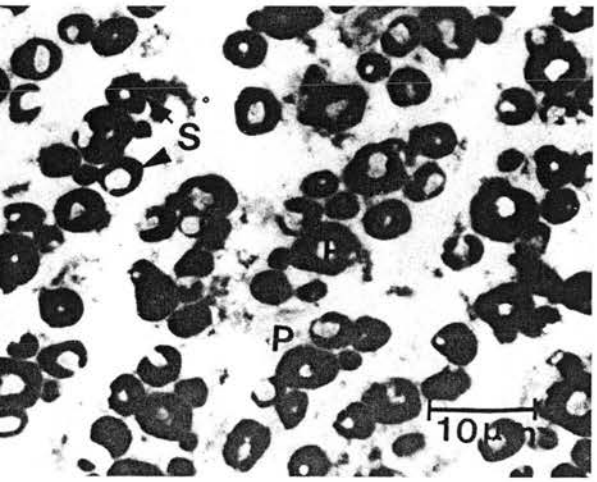
ESULTS

oss Body Parameters. Bone lengths and organ eights (with the exception of brain weight) were gnificantly reduced in the undernourished neo-tes, thus revealing that dietary restriction had en effective. Most parameters diverged in the rd trimester of gestation. Body weight at birth s 40% less in the 60% of ad lib group ($P <$ 001). The lengths of humerus and tibia of the etary restricted group were both reduced at rth by 16% and 18%, respectively.

Muscle Histology. At 30 days of gestation only primary fibers were present in both muscles. The fibers were widely separated and of similar size. At 35 days of gestation in the biceps brachii and soleus muscles, primary and secondary fibers could be differentiated by size and position. The secondary fibers were small and were very closely associated with the "parent" primary fiber. At 40 days of gestation some of the secondary fibers had moved away from their parent fiber and were nearly equivalent in size. Primary and secondary fibers could still be distinguished, however, as primary fibers were at the center of a group of fibers and usually had large myofibrillar free regions (Fig. 2).

At 45 days of gestation (or 50 days in the restricted group) primary and secondary fibers could be easily distinguished by the loss of stability to basic myosin ATPase in the primary fibers in both muscles. However, although this was apparent throughout the soleus muscle (Fig. 3), it was confined to the deep region only in the biceps brachii (Fig. 4). Therefore, at this age, estimates of S/P ratio were confined to the deep region only for biceps brachii.

After 45 days of gestation in the control and 50 days in the restricted muscle, it was no longer possible to distinguish between primary and secondary fibers since there was a uniform distribu-



IGURE 2. Control biceps brachii at 40 days of gestation. BAT-ase histochemical section. P = primary fiber; S = secondary ber.

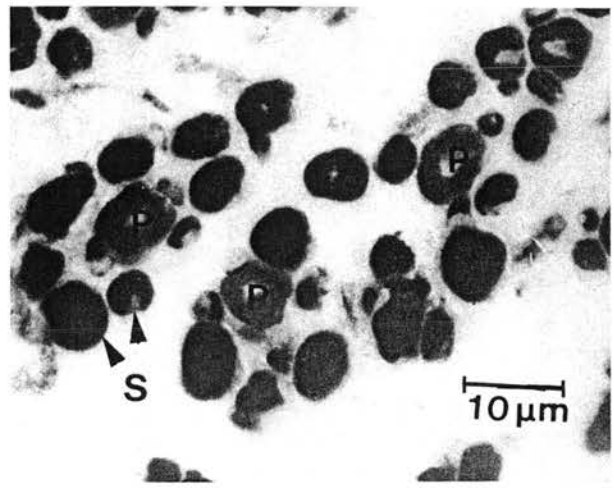


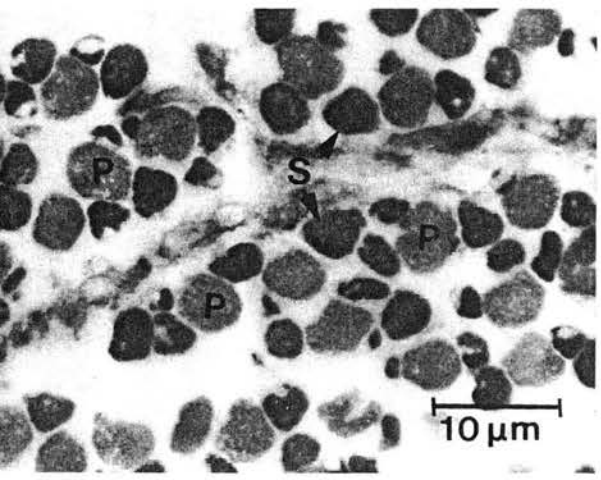
FIGURE 4. Control biceps brachii, deep region, at 45 days of gestation. BATPase histochemical section. P = primary fiber; S = secondary fiber.

on of fiber size and some of the secondary fibers ad converted to a histochemical slow fiber type Fig. 5).

As well as the delayed histochemical matura-on mentioned above, the nutritionally stressed nuscles also displayed morphological immaturity n that myofibrillar free spaces were evident for onger (Fig. 6). Also, average primary fiber size eached a peak at 40 days of gestation in the con-rol muscles compared to 45 days of gestation in ne nutritionally stressed muscle. Following cessa-on of fiber hyperplasia at about 50 days (see be-ow) the fibers grew rapidly in size. The nuclei noved to a peripheral position and myofibrillar assembly accelerated. At all gestational ages muscle fibers of the undernourished animals were always smaller than in the controls.

Fiber Number and Rate of Fiber Formation

Biceps Brachii. Figure 7 shows the increase in muscle fiber number for the ad lib and 60% of ad lib groups. Each point represents the mean for, normally, 6 animals (2 from each of 3 litters). From 30 to 35 days of gestation, fiber number increased slowly and corresponded to the period of primary fiber production. There was no significant difference between the groups at this stage. From 35 to 45 days there was a rapid increase in



IGURE 3. Control soleus at 45 days of gestation. BATPase his-chemical section. P = primary fiber; S = secondary fiber.

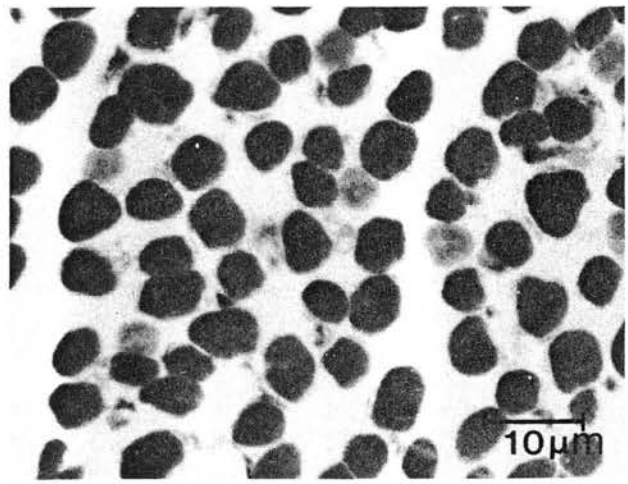


FIGURE 5. Control biceps brachii at 50 days of gestation. BAT-Pase histochemical section.

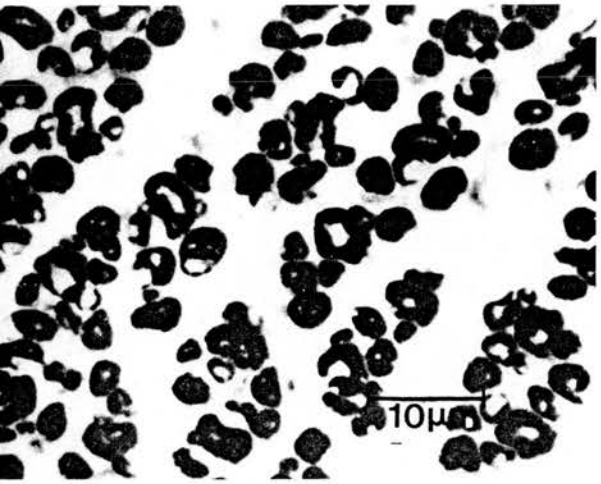


FIGURE 6. Sixty percent of ad lib biceps brachii at 45 days of gestation. BATPase histochemical section.

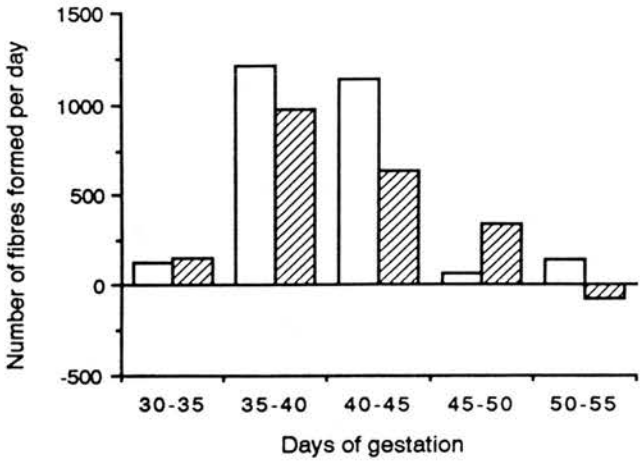
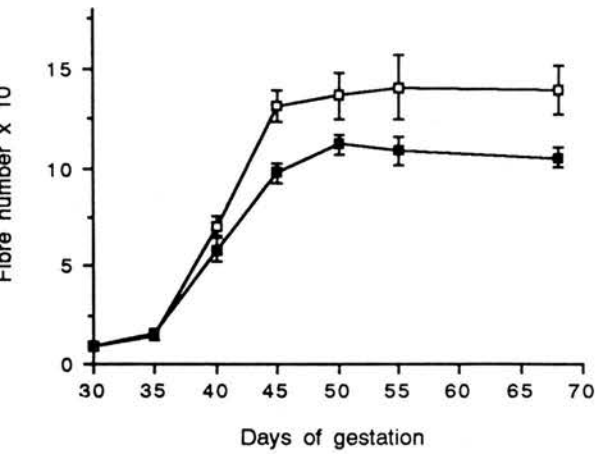


FIGURE 8. Biceps brachii, rate of fiber formation. Ad lib (□); 60% of ad lib (▨). The number of fibers formed per day was calculated from Figure 7.

iber number which was greatest in the control group. The rise in number represented the phase of secondary fiber hyperplasia. While there was no significant increase in fiber number after 45 days in the control, there was a slight but significant increase from 45 to 50 days in the undernourished group. Statistical differences in fiber number between the groups were significant from 40 days of gestation onward. At birth there was a 26% reduction in fiber number in the nutritionally restricted neonates compared to the controls ($P < 0.001$).

From the fiber number data a bar chart was constructed to show the differences in the rate of iber formation between the two groups (Fig. 8). The rate was greater in the ad lib group from 35 to 45 days of gestation. Although from 45 to 50 days of gestation the rate of fiber formation had fallen in both groups, the rate was now relatively higher in the undernourished animals than the controls. From 50 days of gestation onward the rate of fiber formation was very low and there was no apparent difference between the groups.

Soleus. Muscle fiber number was not significantly reduced by the time of birth following prenatal undernutrition (Fig. 9). However, at 45 days of gestation there was a significant difference in fiber number between the groups of about 20% ($P < 0.05$) but this difference disappeared by 50 days. This can be explained by the continuation of



IGURE 7. Change in biceps brachii fiber number with gestational age. Ad lib (□); 60% of ad lib (■). Parturition occurred at pproximately 68 days. Error bars, ±SEM.

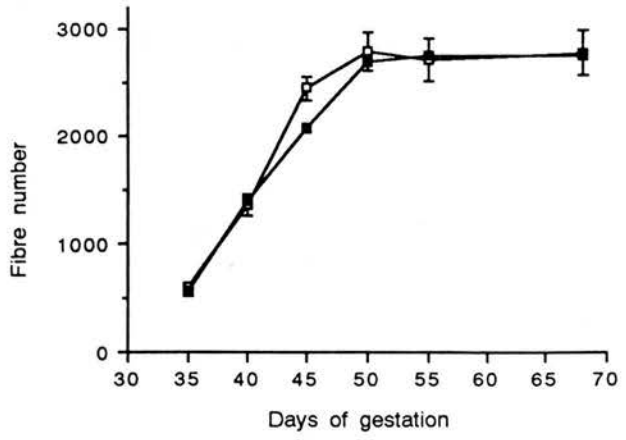


FIGURE 9. Change in soleus fiber number with gestational age. Ad lib (□); 60% of ad lib (■). Error bars, ±SEM.

iber hyperplasia at a higher rate in the restricted group than the control group from 45 to 50 days (Fig. 10).

econdary/Primary Fiber (S/P) Ratios

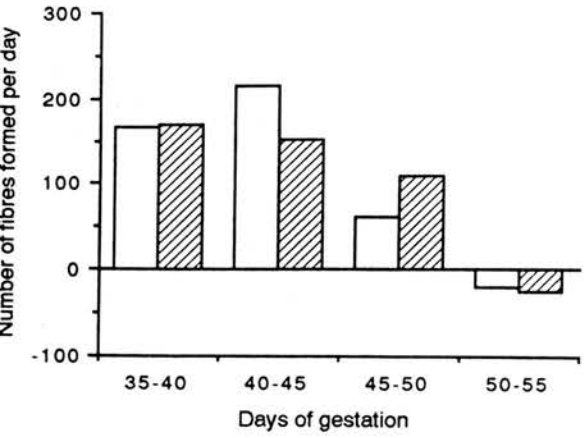
Biceps Brachii. At 40 days the reduction in S/P atio in the dietary restricted muscle was approximately 25% ($P < 0.01$) (Fig. 11). At 45 days of gestation the difference between the groups had increased to 30% ($P < 0.001$). After 45 days S/P atio was impossible to estimate in the control roup since fibers were of similar size and some econdary fibers had begun to histochemically onvert to a pale type. However, as total fiber umber in the controls did not change after this ime then it must follow that S/P ratio was constant. The difference, therefore, between the roups declined by 50 days to approximately 23% ince secondary fiber hyperplasia had been continuing in the undernourished group.

Soleus. S/P ratio was 20% lower ($P < 0.05$) in he undernourished group at 45 days of gestation Fig. 12). There was no difference at any other age.

ISCUSSION

n this study it was found that primary fiber number was unaffected by undernutrition, as there as no difference in fiber number between the utritional groups prior to the onset of secondary ber formation. The reduction in secondary fiber umber was, therefore, entirely responsible for ne reduction in total fiber number at any age.

Muscle fiber number reductions following nutritional restriction in utero have been reported in a number of studies.[1,2,7,13,14] Prior to this study, however, the details of fiber number reduction could only be inferred from work by Wigmore and Stickland[30] and Handel and Stickland.[11] These authors found that fiber number was reduced in small pig littermates compared to their larger siblings and that the cause was a decrease in the number of secondary fibers formed. It is likely that undernutrition was the major factor contributing to the size of the smallest pig siblings.[4,5,9,27] A recent study[31] confirms the results presented here and previously[28] that only the secondary fiber population is affected by nutritional restriction during gestation.



IGURE 10. Soleus, rate of fiber formation. Ad lib (□); 60% of ad b (▨). The number of fibers formed per day was calculated from igure 9.

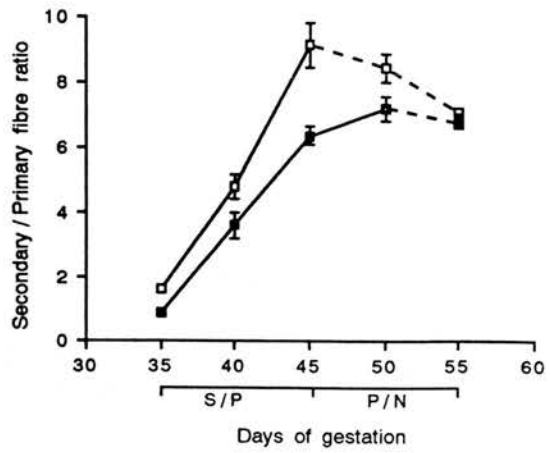


FIGURE 11. Biceps brachii secondary/primary fiber ratio (S/P) and positive-negative basic ATPase fiber ratio (P/N). S/P is shown to 45 days in the control (□) and to 50 days in the 60% of ad lib group (■). P/N is then given by a dashed line.

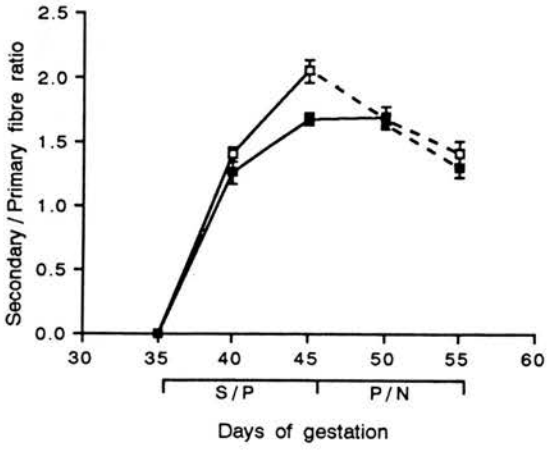


FIGURE 12. Soleus S/P (solid line) and P/N (dashed line). Ad lib (□); 60% of ad lib (■).

There are a number of reasons why primary bers should be spared in nutritional restriction. The first relates to the timing of primary fiber formation which occurs prior to secondary fiber formation when the fetuses are relatively small and making few demands on maternal nutrition. However, different muscles develop at different times. There is a developmental time gradient which runs cephalo-caudally and proximo-distally. Primary fibers in the most distal and caudal muscles would, therefore, be developing when fetuses are large and nutritionally "greedy." However, in the present study the soleus muscle is more distal than the biceps brachii but primary fiber number was still not affected. A second possible reason for the sparing of the primary fiber population could be its relative independence from innervation during development.[19] It has been suggested[24] that nutritional effects on secondary fiber formation are mediated through the nerve supply, yet Wilson et al.[31] were unable to show a nutritional effect on innervation. A third explanation for the maintenance of primary fiber number following undernutrition in utero may be the low rate of primary fiber formation.

In the present study, primary fibers of the biceps brachii developed at a rate of approximately 50 per day compared to 1500 secondary fibers per day. A low plane of nutrition may be able to sustain the slow rate of primary fiber formation but not the faster rate of secondary fiber formation. Furthermore, severe runting in pigs may be associated with a reduction in primary fiber formation, ie, when nutritional restriction is particularly severe.

A decrease in the rate of secondary fiber formation by nutritional restriction could be achieved in one or both of two ways: first, by slowing the rate of myoblast fusion at the surface of the primary fiber, and second, by limiting the availability of myoblasts for fusion by affecting mitosis in that population. Recent work has shown that reduced mitosis is probably the most important factor in the reduction of secondary fiber number following prenatal undernutrition.[18] It was found that nuclear number was reduced to the same extent in both the mononuclear cell population and the muscle fiber population. This suggests that the number of fibers formed is limited by the number of nuclei available from mitotic divisions.

Myoblast fusion may, however, be an important rate-limiting factor in those species with very large numbers of muscle fibers to be formed in a comparatively short period of time. Wigmore and Stickland[30] implied that reduced myoblast fusion was fundamental to the reduction in secondary fiber number in undernourished pig fetuses. The authors hypothesized that the rate of myoblast fusion in undernourished fetuses was limited by the smaller surface area of the primary fibers. This restricted the number of secondary fibers which could form on their surfaces at any given time. There is, in fact, a real difference in the number of newly formed fibers in contact with the primary fiber in large and small pig fetuses.[29] However, the same mechanism is not thought to be operating in rodents, as only 1 or 2 secondary fibers form on the surface of the primary fiber at any one time and subsequent generations probably form in the same place (mouse, Brown; guinea-pig, Madgwick; personal communications).

An important observation in this work was that secondary fibers continued to be formed in the dietary restricted muscle at a higher rate than in the control after 45 days. This continuation of fiber formation is probably an expression of the immaturity of the undernourished muscle. Wilson et al.[31] also found lower maturity in the undernourished lumbricus muscle of the rat. However, they only looked at 2 points during gestation and were, therefore, unable to observe a continuation of secondary fiber production in their restricted animals. Undernutrition during postnatal muscle growth is known to slow the processes of aging.[3,6,20] It appears that undernutrition prenatally delays development in that the cessation of muscle fiber formation is delayed in this study.

Muscle maturity was, in fact, more related to body weight than age; at the same age there was no difference in muscle maturity between the control and restricted groups.

Another important aspect of this work was the difference between the biceps brachii and soleus muscles. There was no difference in fiber number between the undernourished and control soleus muscles from 50 days of gestation onward. There was, however, a period between 40 and 45 days when the number of fibers was significantly lower in the undernourished muscle, and was caused by a reduction in the rate of secondary fiber formation. Subsequently, from 45 to 50 days of gestation, this deficit was corrected by a continuation of fiber formation in the restricted group at a higher rate than in the controls.

The reason why undernutrition had an effect on biceps brachii fiber number and not on soleus fiber number may be related to a difference in S/P

tio between the 2 muscles. By the end of fiber yperplasia in control animals primary fibers con-ibuted approximately 33% of the total in the so-us compared to only 10% in the biceps (Figs. 11 nd 12). As has already been shown, the number f primary fibers in the muscle remained unaf-cted by undernutrition. The period of delayed condary fiber formation in the undernourished leus was able to make up the difference in fiber umber since comparatively less were required to rm the total.

Previous nutritional studies in other species ave shown a reduction in soleus fiber number ut the nutritional regimes were harsher than the resent one (rat: 50% of ad lib through pregnancy nd lactation,[2] 30% of ad lib through pregnancy nd lactation[31]; mouse: protein free through regnancy[1]). In a previous study on undernour-hed guinea-pigs (Ward, unpublished data), a 0% dietary restriction to birth was also found to cause a significant reduction in soleus fiber number. In every case, however, the reduction in fiber number in the soleus muscle was less than the reduction in other, fast twitch, or mixed muscles. From the results presented here it is suggested that the different susceptibilities to nutritional stress of the muscles studied is due to a difference in the proportion of primary fibers.

In conclusion, this study has shown that the reduction in muscle fiber number following undernutrition in utero in the biceps brachii muscle is due to a decrease in the formation of secondary fibers. It is suggested that the soleus muscle (a slow twitch muscle in the adult) was less susceptible to prenatal undernutrition than the putative fast twitch biceps brachii due its higher proportion of primary fibers. Primary fibers were shown to be unaffected by undernutrition. This may be due to their lower and earlier rate of formation compared to the secondary fibers.

EFERENCES

. Aziz-Ullah: Studies on muscle development with particular reference to the effects of protein malnutrition. PhD Thesis, University of Hull, 1974.
. Bedi KS, Birzgalis AR, Mahon M, Smart JL, Wareham AC: Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and refed adult animals. *Br J Nutr* 1982;47:417–431.
. Boreham CAG, Watt PW, Williams PE, Merry BJ, Goldspink G, Goldspink DF: Effects of aging and chronic dietary restriction on the morphology of fast and slow muscles of the rat. *J Anat* 1988;157:111–125.
. Davies DP: Growth of small-for-date babies. *Early Human Dev* 1988;5:95–105.
. Davies DP, Platts P, Pritchard JN, Wilkinson PW: The nutritional status of light-for-date infants at birth and its influence on early postnatal growth. *Arch Dis in Childhood* 1979;54:703–706.
. El Haj AJ, Lewis SEM, Goldspink DF, Merry BJ, Hole han AM: The effects of chronic and acute dietary restriction on the growth and protein turnover of fast and slow muscles of the rat. *Comp Biochem Physiol* 1986;85a:281–287.
. Everitt GC: Prenatal development of uniparous animals with particular reference to maternal nutrition in the sheep, in Lodge GA, Lamming GE (eds): *Growth & Development of Mammals.* London, Butterworths, 1968, pp 137–157.
. Guth L, Samaha FJ: Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Exp Neurol* 1970;28:365–367.
. Hammond J: Physiological factors affecting birth weight. *Proc Nutr Soc* 1944;2:8–14.
. Handel SE: Effects of low birth weight on postnatal development skeletal muscle in the pig. PhD Thesis, University of Edinburgh, 1984.
. Handel SE, Stickland NC: Muscle cellularity and birth weight. *An Prod* 1987;44:311–317.
. Harris AJ: Embryonic growth and innervation of rat skeletal muscle. 1. Neural regulation of muscle fibre numbers. *Phil Trans Roy Soc Lond B* 1981;293:257–277.
13. Howells KF, Jordan TC, Piggott SM: The effects of pre and perinatal restricted food intake on the rat cerebellum. *J Physiol* 1977;273:6–7.
14. Howells KF, Matthews DR, Jordan TC: The effects of pre and perinatal malnutrition on muscle fibres from fast and slow rat muscles. *Res Exp Med* 1977;173:35–40.
15. Kelly AM, Rubinstein NA: Muscle histogenesis and muscle diversity, in Emerson C, Fischman D, Nadal-Ginard B, Siddiqui MAQ (eds): *Molecular Biology of Muscle Development,* Vol 29, Liss New York pp 77–85.
16. Kelly AM, Zacks SI: The histogenesis of rat intercostal muscle. *J Cell Biol* 1969;42:135–153.
17. Kikuchi T: Studies on development and differentiation of muscle. III Especially on the mode of increase in the number of muscle cells. *Tohoku J Agric Res* 1971;22:1–15.
18. Madgwick AJA, Stickland NC: The effect of undernutrition on nuclear populations in fast and slow twitch muscles of the guinea pig. *J Anat,* in press.
19. McLennan IS: Neural dependence and independence of myotube production in chicken hindlimb muscles. *Dev Biol* 1988;98:287–294.
20. Merry BH, Holehan AM: The endocrine response to dietary restriction in the rat, in Woodhead AD, Blackett AD, Hollaender A (eds): *Molecular Biology of Ageing,* Basic Life Science 35, New York/London, Plenum, 1985, pp 117–141.
21. Ontell M: Neonatal muscle: An electron microscopic study. *Anat Rec* 1977;189:669–690.
22. Ontell M, Dunn RF: Neonatal muscle growth; a qualitative study. *Am J Anat* 1978;152:539–556.
23. Peter JB, Barnard RJ, Edgerton VR, Gillespie CA, Stempel KC: Metabolic profiles of 3 types of skeletal muscle in guinea-pigs and rabbits. *Biochem* 1972;11:2627–2633.
24. Ross JJ, Duxson MJ, Harris AJ: Neural determination of muscle fibre numbers in embryonic rat lumbrical muscle. *Development* 1987;100:359–409.
25. Stickland NC, Widdowson EM, Goldspink G: Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscles of pigs. *Br J Nutr* 1975;34:421–428.

Swatland HJ, Cassens RG: Inhibition of muscle growth in foetal sheep. *J Agric Sci Camb* 1975;80:503–509.
Walther FJ, Ramaekers JMA, Van Engelshoven P: Skeletal maturity at birth and at the age of 3 years of infants malnourished in utero. *Early Hum Dev* 1981;5:139–143.
Ward SS, Stickland NC: The effect of undernutrition on fibre number in myogenesis. *1987;155:240.*
29. Wigmore PMC: Prenatal muscle development in the pig. PhD Thesis, University of Edinburgh, 1982.
30. Wigmore PMC, Stickland NC: Muscle development in large and small pig foetuses. *J Anat* 1983;137:235–245.
31. Wilson SJ, Ross JJ, Harris AJ: A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats. *Development* 1988;102:815–821.

The effect of undernutrition in the early postnatal period on skeletal muscle tissue

BY STEPHANIE S. WARD AND NEIL C. STICKLAND

Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU

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Guinea-pigs were undernourished from birth by first, cross-fostering in groups of six (in pairs for control animals) and then by feeding a diet of 60% of *ad lib.* intake from 2 to 6 weeks. The changing characteristics of muscle fibre types in the *biceps brachii* and *soleus* muscles were monitored by histochemistry. Undernutrition appeared to delay maturation of muscle fibres. Fibre cross-sectioned area was reduced in all fibres of the 60% of *ad lib.* intake group. Fibres of the *biceps brachii* were more affected than those of the *soleus*. Fibre area vulnerability was partly associated with a high relative growth rate in *biceps brachii* at this time. Total protein content in the *semitendinosus* muscle was depressed in the undernourished group compared with the control group. DNA concentrations were initially higher in the control group but decreased in both groups to similar levels by 6 weeks. RNA concentration increased up to 5 weeks in the control group and then decreased, but decreased after 2 weeks in the undernourished group.

Muscle: Undernutrition: Differentiation

In the early postnatal period muscles are transformed from immature into efficiently adapted units. Expression of neonatal and foetal myosin isoforms are suppressed while adult ones are enhanced (Gauthier *et al.* 1978, 1982). The increased sarcomere-shortening velocity in fast-twitch muscles, which is correlated with calcium-activated ATPase (*EC* 3.6.1.32) (Barany, 1967), leads to a concurrent rise in glycolytic metabolism and a decrease in oxidative metabolism (Goldspink, 1962; Baldwin *et al.* 1978). Slow-twitch muscles, on the other hand, become increasingly oxidative and retain low sarcomere-shortening velocities (Close, 1964; Drachman & Johnson, 1973). Decrease in fast-type myosins can be monitored by the loss of stability at an alkaline pH in myosin ATPase histochemistry (Goldspink & Ward, 1979).

Alterations in metabolic profile are thought to be mediated through fibre size (Goldspink & Howells, 1974; Swatland, 1983). Myosin transformations are the result of nervous intervention (Engel & Kaparti, 1968; Butler-Browne & Whalen, 1984) and hormonal signalling, particularly via thyroxine (Baldwin *et al.* 1978; Ianuzzo *et al.* 1980). Conditions which upset the normal controlling processes would be expected to disrupt the course of differentiation in the muscle.

The effect of undernutrition on mature muscle (i.e. following the fibre hyperplastic phase) has generally been found to be reversible (Goldspink & Ward, 1979). The main effect is a reduction in fibre size (Stickland *et al.* 1975; Goldspink & Ward, 1979) which is mediated by a depression in protein synthesis (Winick & Nobel, 1966). The proportion of fibre types has been found to be unaffected by undernutrition imposed on mature animals (Goldspink & Ward, 1979). In animals experiencing dietary restriction prenatally, however, there is evidence that fibre-type proportions may be altered in the adult (Howells *et al.* 1978; Bedi

et al. 1982). The effect of dietary restriction on skeletal muscle tissue in the early postnatal period has not been comprehensively studied. The present study was designed to investigate the possibility that undernutrition confined to the period of postnatal muscle differentiation may affect the transitional maturation of muscle fibre types.

MATERIALS AND METHODS

Animals

Thirty-six neonatal guinea-pigs (weighing over 50 g) and lactating females were obtained from Tuck and Son, Battlesbridge, Essex. The animals were divided into two equal groups. The first was fostered in pairs with one lactating female, and the second was fostered in groups of six per female. This procedure ensured that the second group was undernourished compared with the first group from birth to weaning.

Weaning for all animals was at 2 weeks postnatally at which time the young were housed separately and fed on solid food. The group which had been in large lactation units was restricted to a diet of 60% of *ad lib.* intake which was measured daily against the other group, the individuals of which acted as pair-fed controls. The diet used was the commercial SG1 pellet (Quest Nutrition, Canterbury, Kent). The diet was supplemented with vitamin E (Roche) at a concentration of 60 mg α-tocopherol/kg diet. Three animals from each group were killed at weekly intervals from 1 to 6 weeks of age (using an intraperitoneal injection of sodium pentobarbitol). Various body measurements were taken including weights of body, liver and brain and lengths of humerus and tibia. From each animal *biceps brachii*, *soleus* and *semitendinosus* muscles were removed, weighed and used for further analysis.

Histochemistry

The *biceps brachii* and *soleus* muscles were chosen for histochemical analysis since they represent fast- and slow-twitch muscles respectively. Frozen sections (10 μm) were taken from the mid-belly of each muscle and processed for the following three histochemical techniques: (1) myosin ATPase (*EC* 3.6.1.32) with pre-incubation at either pH 10·6 (BATPase) or pH 4·6 (AATPase) (Guth & Samaha, 1970), (2) succinate dehydrogenase (SDH; *EC* 1.3.99.1) (Nachlas *et al.* 1957) and (3) glycogen phosphorylase (GP; *EC* 2.4.1.1) (Takeuchi, 1956). Fibres were classified as positive or negative (and, in some cases, intermediate) for each reaction. Proportional counts of fibre 'types' were based on analysis of approximately 100 fibres for each muscle sample. Samples were also taken from three newborn animals for this analysis.

Fibre size

Fibre area estimations were made on frozen sections using 100 fibres of each type and from each muscle. Measurements were performed on a digital pad attached to a computer package (VIDS; Analytical Measuring Systems Ltd, Saffron Walden, Essex).

Biochemistry

Protein and nucleic acid content was estimated for the *semitendinosus* muscles. Samples were taken from the middle two-thirds of the muscle.

RNA and DNA. Nucleic acids were extracted using a modified Schmidt Thannauser method (Munro & Fleck 1966), with RNA and DNA concentrations being calculated from absorbance measurements at wavelengths 260 and 268 nm respectively.

Protein. Concentrations were estimated by the method of Wigmore (1982), based on those of Helander (1957) and Millward (1970). Protein was separated into sarcoplasmic

and fibrillar fractions using salt extraction. A commercial dye reagent (Biorad) was added to the extractions and readings were taken from a spectrophotometer (LKB) at 595 nm. Protein concentration was then calculated using a standard curve of bovine serum albumin.

RESULTS

Gross body variables

Measurements on gross body variables served to illustrate that both the cross-fostering technique (up to 2 weeks) and the 60% of *ad lib.* intake dietary regimen (2–6 weeks) was exerting a significant effect on body growth (Fig. 1). Body weight was increasingly affected in the undernourished animals over the 6 week period. By 6 weeks the percentage difference from the control was 35 (Student's t test; $P < 0{\cdot}05$). Liver weight was reduced by 40% ($P < 0{\cdot}001$), whereas brain weight was not significantly different. Bone lengths were also reduced (tibia by 11% and humerus by 15%).

Muscle fibre size

Biceps brachii. A paired t test revealed that, on average, the positive BATPase fibres were 46% smaller ($P < 0{\cdot}001$) and the negative BATPase fibres were 35% ($P < 0{\cdot}005$) smaller in the undernourished group.

Soleus. Both BATPase positive and negative fibres were on average 27% smaller in the 60% of *ad lib.*-fed animals ($P < 0{\cdot}01$).

It is thought that the degree to which tissues and organs are affected by undernutrition is dependent on their relative growth rate at the time of dietary restriction. For this reason logarithmic regressions of fibre area *v.* body weight were calculated. If fibre area (Y) is increasing in line with body weight (X) then the expected relationship is $Y = K.X^{0{\cdot}66}$. The regression coefficients were, therefore, compared with 0·66 (Table 1).

It can be seen from Table 1 that both muscles studied had a high relative growth rate in *ad lib.* situations. This growth rate was largely reduced by restricted nutrition. The implication from this finding is that muscle tissue is more affected by undernutrition than overall body weight.

Histochemistry

Biceps brachii. (*1*) *Myosin ATPase.* The number of negative BAPTPase fibres was counted in the deep region of the muscle and expressed as a percentage of the total number in the deep area. There was no change in the ratio of fibres with age and there was no significant difference between the groups (*ad lib.* 12·2%, 60% of *ad lib.* intake 12·4%).

(*2*) *SDH.* Fibres were classified as positive, intermediate or negative for the reaction. In the superficial area of the muscle all fibres were positive until 2 weeks of age in the *ad lib.*-fed group and 4 weeks in the undernourished when there was a swift transition to 60% negative in the *ad lib.*-fed animals (Plate 1) and 55% in the restricted group. The difference between the two groups was not significant.

In the deep area of the muscles the number of positive fibres remained constant with age and formed about 75% in both groups. The remaining 25% were intermediate fibres until 3 weeks in the *ad lib.* and 5 weeks in the restricted group. About half the intermediate fibres then became negative leaving approximately 12% intermediate fibres in the deep area of the muscle. These intermediate fibres corresponded to the negative BATPase fibres in this area. They could now be classified as slow oxidative (Peter *et al.* 1972).

(*3*) *GP.* GP staining was inversely related to SDH staining. There was no staining at all for the initial postnatal period until the appearance of the first SDH negative fibres. The biceps then rapidly became increasingly GP positive.

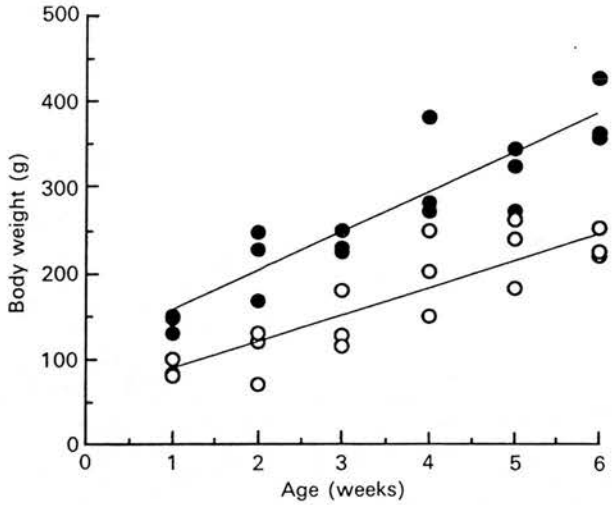

Fig. 1. Body weights of all guinea-pigs used in the study. Computed regression lines are shown for each group. (●), Control group; (○), 60% of *ad lib.*-fed group. For details of dietary regimen, see p. 142.

Table 1. *Regression coefficients for the relationship between fibre cross-sectioned area and body weight in guinea-pigs fed* ad lib., *or at 60% ad lib. intake**

(Mean values with their standard errors)

Muscle fibre type	*b*				Statistical significance of difference between groups: $P <$	Statistical significance of difference from 0·66: $P <$	
	Ad lib.		60% *ad lib.*				
	Mean	SE	Mean	SE		*Ad lib.*	60% of *ad lib.*
Biceps							
BATPase+	0·94	0·3	0·92	0·1	NS	0·025	0·005
BATPase−	1·23	0·2	0·65	0·4	0·001	0·001	NS
Statistical significance of difference between BATPase+ and BATPase−: $P <$	0·05		0·05				
Soleus							
BATPase+	1·0	0·4	0·61	0·27	0·01	0·001	NS
BATPase−	0·85	0·19	0·5	0·26	0·005	0·05	NS
Statistical significance of difference between BATPase+ and BATPase−: $P <$	0·05		NS				

BATPase, myosin ATPase (*EC* 3.6.1.32) pre-incubated at pH 10·6; NS, not significant.
* For details of dietary regimen, see p. 142.

Soleus. (*1*) *Myosin ATPase*. The *soleus* had a mixed population of fibres at birth in terms of their staining with myosin ATPase. The number of positive BATPase fibres was constant at approximately 45% between birth and 2 weeks of age and then fell steadily to 6 weeks postnatally (Fig. 2). There was no difference between the nutritional groups in the rate of

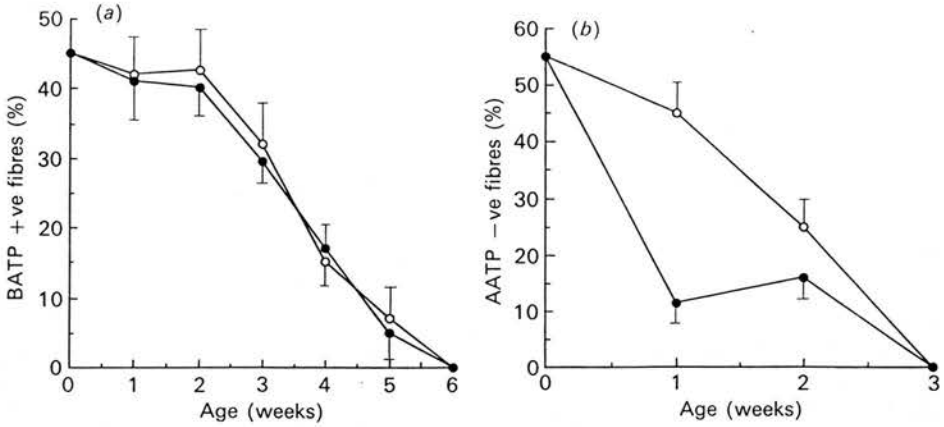

Fig. 2. Disappearance of immature reactions with ATPase (*EC* 3.6.1.32) histochemistry in the *soleus* muscle of guinea-pigs. (*a*) Positive ATPase pre-incubated at pH 10·6 (BATPase) fibres; (*b*) negative ATPase pre-incubated at pH 4·6 (AATPase) fibres. Points represent the means with their standard errors represented by vertical bars for three animals. (●), Control; (○), 60% of *ad lib.*-fed group. For details of dietary regimen, see p. 142.

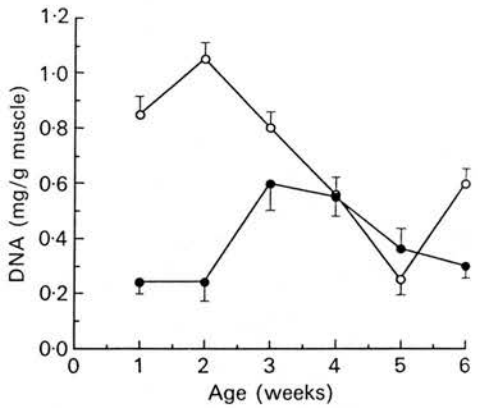

Fig. 3. Concentration of DNA extracted from the *semitendinosus* muscle of guinea-pigs. Points represent the means with their standard errors represented by vertical bars for three animals. (●), Control; (○), 60% of *ad lib.*-fed group. For details of dietary regimen, see p. 142.

disappearance of these fibres. Negative AATPase fibres also fell with age, such that from an average of 55% at birth there were none by 3 weeks postnatally (Fig. 4). Initially the number fell more rapidly in the control group so there were less AATPase negative fibres than the undernourished muscle at 1 week ($P < 0{\cdot}001$). At 2 weeks, however, the number of AATPase negative fibres was similar in the two groups.

The immature fibres were the smallest in the muscle so that the negative AATPase and positive BATPase populations were overlapping. The adult muscle was 100% BATPase negative and AATPase positive.

(*2*) *SDH*. There was no change in the SDH staining pattern with age. In both groups fibres remained 100% intermediate.

(*3*) *GP*. The muscle fibres were very weakly positive and corresponded to the number of BATPase positive fibres present.

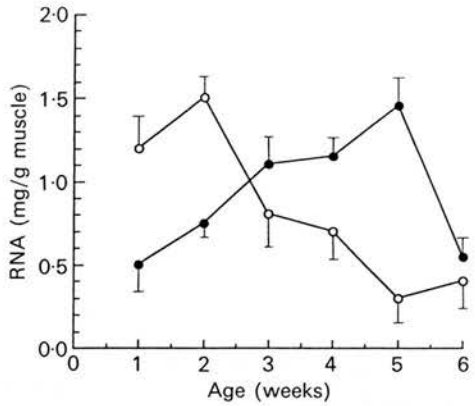

Fig. 4. Concentration of RNA extracted from the *semitendinosus* muscle of guinea-pigs. Points represent means with their standard errors represented by vertical bars for three animals. (●), Control; (○), 60% of *ad lib.*-fed group. For details of dietary regimen, see p. 142.

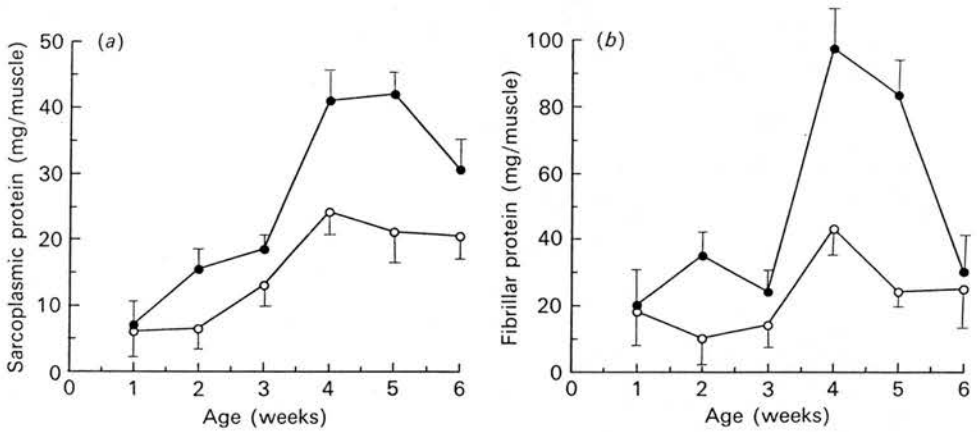

Fig. 5. Protein extracted from the *semitendinosus* muscle. (*a*) Total fibrillar protein per muscle; (*b*) total sarcoplasmic protein per muscle. Points represent the means with their standard errors represented by vertical bars for three animals. (●), Control; (○), 60% of *ad lib.*-fed group. For details of dietary regimen, see p. 142.

Biochemistry

Nucleic acids and protein fractions were expressed as concentrations throughout the postnatal period under study. Protein fractions were also expressed as total amounts per muscle. The results are shown in Figs. 3–5. Generally, the tendency in control animals was for concentration of all variables to increase initially during growth with DNA declining after about 4 weeks (Fig. 3), and all other variables declining after 5 weeks (Figs. 4 and 5).

In the 60% of *ad lib.*-fed group, concentrations of DNA and RNA fell with age after 2 weeks (Figs. 3 and 4). There was no demonstrable difference between the nutritional groups in protein concentrations but total amounts of both sarcoplasmic and fibrillar fractions were reduced in the undernourished muscles (Fig. 5). Ratios that have been commonly used to reveal nutritional effects on variables associated with protein synthesis were calculated for each age. Differences were investigated using a paired t test. The results (Table 2) showed a difference between nutritional groups for RNA:DNA and for protein:DNA ratios but not for the sarcoplasmic:fibrillar protein ratios. None of the ratios showed any

Table 2. *Mean values for ratios of biochemical variables associated with protein synthesis in guinea-pigs fed* ad lib. *or at 60% of* ad lib. *intake**

(Mean values with their standard errors)

	RNA:DNA		Protein:DNA		Sarcoplasmic: fibrillar protein	
Dietary regimen	Mean	SE	Mean	SE	Mean	SE
Ad lib.	2·3	0·01	468	12	0·58	0·001
60% *ad lib.*	1·3	0·2	283	9	0·7	0·001
Statistical significance of difference: $P <$	0·005		0·001		NS	

NS, not significant.

* For details of dietary regimen, see p. 142.

marked change with age. The values given in Table 2 mean values for the entire period under study.

DISCUSSION

It was clear from the results of the present study that undernutrition acted on the muscles by delaying some processes of differentiation. Aspects of fibre histochemistry which had been established before birth, such as myosin ATPase in the *biceps brachii* and SDH in the *soleus* muscles, were unaffected.

The metabolic profile of the *biceps brachii* changed in the perinatal period. Changes in the 60% of *ad lib.*-fed group were delayed by approximately 2 weeks. The controlling mechanism for fibre transitions is thought to be weight specific (Ashmore *et al.* 1972) and is supported by the work presented here. At 2 and 3 weeks in the control and 4 and 5 weeks in the dietary-restricted groups (which is when the transitions occurred) there were no significant differences in body weights.

Goldspink & Howells (1974) suggested that fibre size was the main determinant of metabolic type. A fibre grows by proliferation of sarcoplasmic and myofibrillar material which increasingly dilutes the mitochondria. They are no longer able to survive on the reduced O_2 levels available by diffusion across the cell surface. O_2 levels are further eroded by the now inadequate capillary bed (Sillau & Banchero, 1977). Energy demands are met by a switch to glycolysis, the enzymes for which multiply rapidly. There is, therefore, an inverse relationship between oxidative and glycolytic metabolism (Dubowitz & Pearce, 1960).

In Table 1 it can be seen that fibre area and body-weight were related. Undernutrition might, therefore, delay metabolic enzyme alterations via body weight. While this explanation holds for the *biceps brachii*, it seems from the *soleus* muscle that there was no direct relationship between fibre size and metabolic type. For example, the smallest fibres in the *soleus* were glycolytic. At the point of conversion (which occurred at the same time in *soleus* of the two nutritional groups), fibres in the undernourished groups were always smaller than those in the control muscle. The relationship between fibre size and enzyme transformations (at least during differentiation) may be purely a reflection of the immaturity of smaller fibres. The smallest fibres in the *soleus* muscle were also those with immature myosin ATPase characteristics.

The adult guinea-pig *soleus* is 100% myosin ATPase acid stable and alkaline labile. In the present study it was found that AATPase lability was lost before BATPase lability was

gained in some of the fibres. Alkaline stability is bestowed by fast myosin alkaline light chain 1 and acid stability by slow alkaline light chain 1 (Baldwin, 1984). This suggests that for the period of a few weeks slow and fast myosin types were coexistent in the same fibres. Before the attainment of acid stability at 3 weeks postnatally, some of the fibres were only expressing a fast type of isozyme. Undernutrition did not affect the rate of elimination of basic stability, i.e. the disappearance of fast myosin types (which may have been adult or neonatal). On the other hand, it seemed that there was an effect on the appearance of the mature slow myosin isoform in the undernourished animal.

In the rat *soleus* muscle, from a prenatal expression of four foetal and neonatal (fl-4) and two adult slow isozymes (SM1 and SM2) which differ in both light and heavy chains, there is a progressive elimination of all but one of the two adult slow isozymes (SM2) (Lyons *et al.* 1983). In the present study the number of AATPase negative fibres rose in the control muscle at 2 weeks postnatally. This suggested that a slow myosin isoform was briefly lost in some fibres. This might occur by a slight delay between SM1 being suppressed and SM2 being produced in a few of the fibres. For a while, therefore, there was no slow myosin in individual fibres which would be acid labile. In the dietary-restricted *soleus* muscle there was no such decline in acid-stable fibres. This could be explained simply by SM2 being manufactured before SM1 decreased, i.e. the elimination of SM1 was delayed in the undernourished animals.

It is thought that the progression of fast-type isoenzymes is controlled by thyroid hormone and slow ones by the intervention of tonic innervation (Butler-Browne & Whalen, 1984). However, it is known that fibres begin differentiating while still polyneuronally innervated (Hoh *et al.* 1988). Unless nervous input is homogeneous there must be a control which ensures that only one nerve type (i.e. slow or fast twitch) is perceived by the muscle fibre. It has been suggested that load directs fibres to express slow myosin isoforms (Noble *et al.* 1983). Delaying effects of undernutrition on the appearance of adult slow myosin isoforms might, therefore, be associated with a reduction in body weight.

There was an effect of undernutrition on all biochemical variables measured. Although there must be a gradual increase in total DNA in a given muscle due to satellite cell proliferation (Moss & Leblond, 1971), a decrease in DNA concentration reflects growth in cell size or tissue volume (Prior *et al.* 1979; Wigmore, 1982). The general tendency in the present study was for DNA concentration to fall with age after 2–3 weeks, except initially when total DNA was rapidly increasing. The concentration of DNA in the undernourished muscle was higher than that of the control because the cells were smaller. Later, although there was no difference in DNA concentrations between the groups, this is clearly not due to equivalence in fibre size between the groups (note the effects on fibre size in the *biceps* and *soleus* muscles). It has already been shown that fibre size reduction is greater than muscle cross-sectioned area reduction in undernourished muscles (Ward, 1989). The extracellular compartment must have diluted the DNA fraction in the malnourished animal to the same extent that increased fibre size reduced concentrations in the control muscle.

RNA concentration increased with age in the control group, whereas in the malnourished muscle it decreased. The low rate of RNA increase in the undernourished *semitendinosus* was not sufficient to offset the reduction in RNA concentration which would occur with increase in muscle size. Not only was there an apparent effect of undernutrition on transcription but there also appeared to be a reduction in translation. This was seen in the lower total levels of protein in the malnourished muscle.

There was no difference in the extent to which the fibrillar and sarcoplasmic proteins were affected by postnatal malnutrition. Prenatally the structural proteins are more at risk (Aziz-Ullah, 1974; Wigmore, 1982) which leads to a rise in the sarcoplasmic:fibrillar ratio. The difference in the developmental periods is presumably related to the diverse activity of

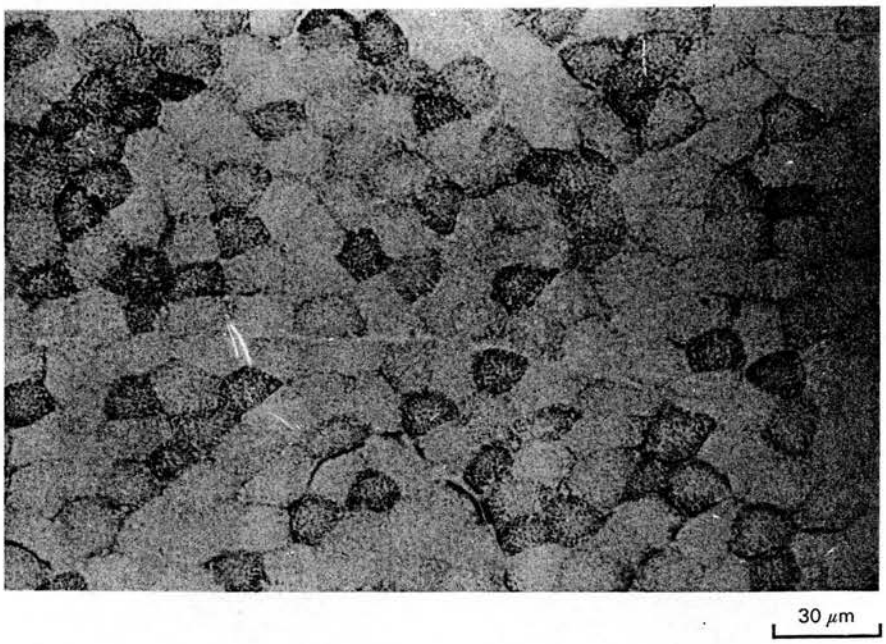

Plate 1. Superficial area of the control guinea-pig *biceps brachii* reacted for succinate dehydrogenase (*EC* 1.3.99.1) at 6 weeks of age. For details of procedures, see p. 142.

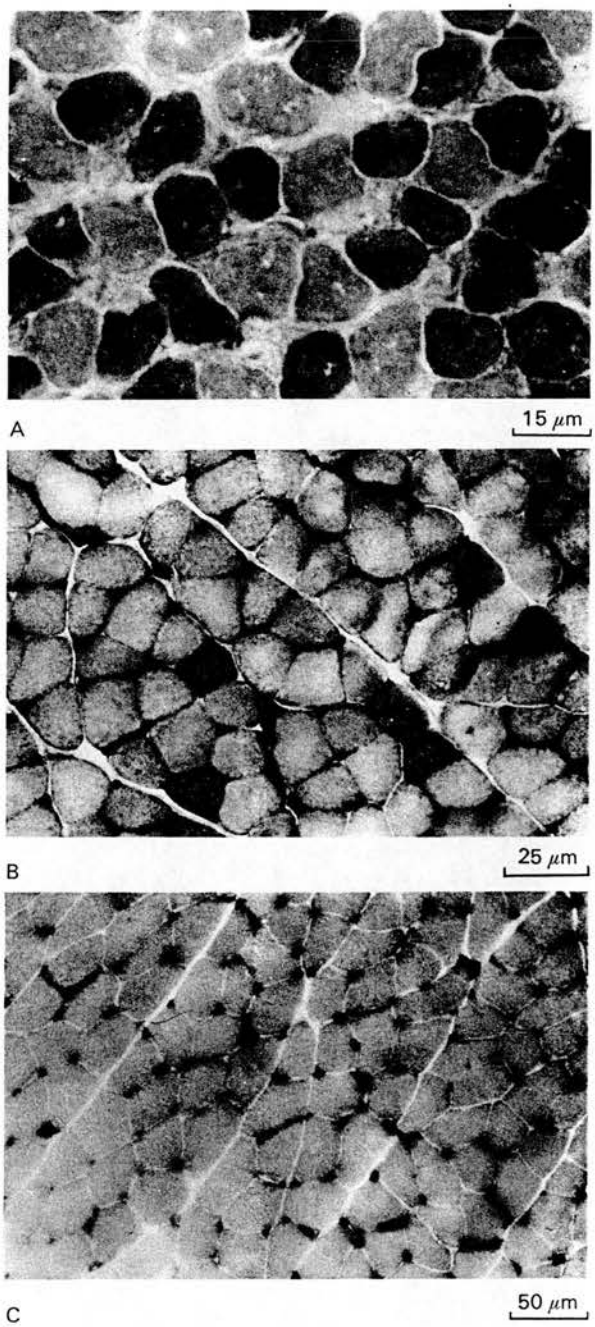

Plate 2. Control guinea-pig *soleus* reacted for ATPase (*EC* 3.6.1.32) in a basic medium. (A), neonatal; (B), 5 weeks; (C), 6 weeks. For details of procedures, see p. 142.

the muscle fibres at these times. The fibres are less contractile before birth so the metabolic proteins are preserved at the expense of the myofilaments. Postnatally both components are equally important and their roles are interdependent.

RNA concentration in the control *semitendinosus* muscle exhibited a change at about 5 or 6 weeks. This is the watershed between the rapid growth phase and a slower growth period. At 5 weeks postnatally guinea-pig weight is only half its adult level, so that maximum muscle size is still to be achieved. However, in the intervening growth period to maturity protein synthesis must be occurring at a lower rate than previously. The greatest increase is sustained by lower rates of protein degradation. Furthermore, 5 weeks may mark the end of a developmental process. The *semitendinosus* muscle has a mixed-fibre-type population, with many slow oxidative fibres in the deep region of the muscle. It is possible that enzyme transitions similar to those in the *soleus* muscle were occurring. These enzyme changes were complete by 5–6 weeks postnatally. The achievement of metabolic maturity might coincide with a decline in RNA levels at this time.

To conclude, it has been shown that undernutrition delays the process of metabolic enzyme conversion in the fast-twitch *biceps brachii*. Myosin ATPase reactions were already mature by the time of birth and were not affected by inadequate nutrition. Conversely, in the soleus muscle there were effects on myosin ATPase properties which can be explained by the fact that these properties were maturing up to 6 weeks postnatally in this muscle. Some of the effects were due to reductions in fibre area, although it was apparent that size was not the only cause. The depression in RNA concentrations and protein:DNA ratios in the 60% of *ad lib.*-fed group points to lowered rates of protein synthesis in these animals.

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REFERENCES

Ashmore, C. R., Addis, P. B. & Doerr, L. (1972). Postnatal development of muscle fibre types in domestic animals. *Journal of Animal Science* **34**, 37–41.

Aziz-Ullah (1974). Studies on muscle development with specific reference to the effects of protein malnutrition. PhD Thesis, University of Hull.

Baldwin, K. M (1984). Muscle development; neonatal to adult. *Exercise and Sport Sciences Reviews* **12**, 1–19.

Baldwin, K. M., Cambell, P. J., Hooker, A. M. & Lewis, R. E. (1978). Enzyme changes in neonatal skeletal muscle; effect of thyroid deficiency. *American Journal of Physiology* **235**, 97–102.

Barany, M. (1967). ATPase activity of myosin correlated with speed of shortening. *Journal of General Physiology* **50**, 197–218.

Bedi, K. S., Birzgalis, A. R., Mahon, M., Smart, J. L. & Wareham, A. C. (1982). Early life undernutrition in rats; I. Quantitative histology of skeletal muscles from underfed young and adult refed animals. *British Journal of Nutrition* **47**, 417–431.

Butler-Browne, G. S. & Whalen, R. G. (1984). Myosin isozyme transitions occurring during the postnatal development of the rat soleus muscle. *Developmental Biology* **102**, 324–334.

Close, R. (1964). Dynamic properties of fast and slow muscles of the rat during development. *Journal of Physiology* **173**, 74–95.

Drachman, P. B. & Johnson, D. M. (1973). Development of a mammalian fast muscle; dynamic and biochemical properties related. *Journal of Physiology* **234**, 29–43.

Dubowitz, V. & Pearce, A. G. E. (1960). Reciprocal relation of phosphorylase and oxidative enzymes in skeletal muscle. *Nature* **185**, 701–702.

Engel, W. K. & Kaparti, G. (1968). Impaired skeletal muscle maturation following neonatal neurectomy. *Developmental Biology* **17**, 713–723.

Gauthier, G. F., Lowey, S., Benfield, P. A. & Hobbs, A. W. (1982). Distribution and properties of myosin isozymes in developing avian and mammalian skeletal muscle fibres. *Journal of Cell Biology* **92**, 47–84.

Gauthier, G. F., Lowey, S. & Hobbs, A. W. (1978). Fast and slow myosin in developing muscle fibres. *Nature* **274**, 25–29.

Goldspink, G. (1962). Biochemical and physiological changes associated with the postnatal development of the biceps brachii. *Comparative Biochemistry and Physiology* **7**, 157–168.

Goldspink, G. & Howells, K. F. (1974). Work induced hypertrophy in exercised normal muscles of different ages and the reversibility of hypertrophy after the cessation of exercise. *Journal of Physiology* **239**, 179–193.

Goldspink, G. & Ward, P. S. (1979). Changes in rodent muscle fibre types during postnatal growth, nutrition and exercise. *Journal of Physiology* **296**, 453–469.

Guth, L. & Samaha, F. J. (1970). Research note: Procedure for the histochemical demonstration of Actomyosin ATPase. *Experimental Neurology* **28**, 365–367.

Helander, E. (1957). On quantitative muscle protein determination. Sarcoplasm and myofibril protein content of normal and atrophic skeletal muscles. *Acta Physiologica Scandinavica* **41**, Suppl., 141.

Hoh, J. F., Hughes, S., Hugh, G. & Pozagaj, I. (1988). Three hierarchies in skeletal muscle fibre classification: allotype, isotype and phenotype. *UCLA Symposia on Molecular and Cellular Biology* Suppl., 12C.

Howells, K. F., Matthews, D. R. & Jordan, T. C. (1978). Effects of pre and perinatal malnutrition on muscle fibres from fast and slow rat muscles. *Research into Experimental Medicine* **173**, 35–40.

Ianuzzo, C. P., Patel, P., Chen, V. & O'Brien, P. (1980). A possible thyroidal trophic influence on fast and slow skeletal myosin. In *Plasticity of Muscle*, pp. 593–606 [D. Pette, editor]. Berlin: de Gruyter.

Lyons, S. E., Kelly, A. M., Rubenstein, N. A. & Hazelgrove, J. (1983). Myosin transitions in developing fast and slow muscles of the rat hind limb. *Differentiation* **25**, 163–175.

Millward, D. J. (1970). Protein turnover in skeletal muscle. I. The measurement of rates of synthesis and catabolism of skeletal muscle protein using (^{14}C) Na_2CO_3 to label protein. *Clinical Sciences* **39**, 577–590.

Moss, F. P. & Leblond, C. P. (1971). Satellite cells as the source of nuclei in the muscle of growing rats. *Anatomical Record* **170**, 421–430.

Munro, H. N. & Fleck, A. (1966). The determination of nucleic acids. *Methods of Biochemical Analysis* **14**, 133–176.

Nachlas, M. M., Tsouk, K., De Sousa, F., Cheng, C. & Seligman, M. (1957). Cytochemical demonstration of succinic dehydrogenase by the use of a new *p*-nitrophenol substituted ditetrazole. *Journal of Histochemistry and Cytochemistry* **5**, 420–436.

Noble, E. G., Dabrowski, B. L. & Ianuzzo, C. P. (1983). Myosin transformation in hypertrophied rat muscle. *Pflügers Archives* **396**, 260–271.

Peter, J. B., Barnard, R. J., Edgerton, V. R., Gillespie, C. A. & Stempel, K. E. (1972). Metabolic profiles of 3 fibre types of skeletal muscle in guinea-pigs and rabbits. *Biochemistry* **11**, 2627–2633.

Prior, R. C., Scott, R. A., Lister, D. B. & Campion, D. R. (1979). Maternal energy status and development of liver and muscle in the bovine foetus. *Journal of Animal Science* **48**, 1534–1545.

Sillau, A. H. & Banchero, N. (1977). Effect of malnutrition on capillary density, fibre size and composition in rat skeletal muscle. *Proceedings of the Society of Experimental and Biological Medicine* **154**, 461–466.

Stickland, N. C., Widdowson, E. M. & Goldspink, G. (1975). Effects of severe energy and protein deficiencies on the fibres and nuclei of skeletal muscle of pigs. *British Journal of Nutrition* **34**, 421–428.

Swatland, H. J. (1983). Aerobic activity in the axis of the growing myofibres in the porcine biceps femoris. *Journal of Animal Science* **56**, 1324–1328.

Takeuchi, T. (1956). Histochemical demonstration of phosphorylase. *Journal of Histochemistry and Cytochemistry* **4**, 84.

Ward, S. S. (1989). The effect of undernutrition on skeletal muscle development and growth in the guinea pig. PhD Thesis, University of London.

Wigmore, P. M. C. (1982). Prenatal muscle development in the pig. PhD Thesis, University of Edinburgh.

Winick, M. & Nobel, A. (1966). Cellular response in rats during malnutrition at various ages. *Journal of Nutrition* **89**, 300–306.

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ort Report

oes the anatomical location of a muscle affect the influence undernutrition on muscle fibre number?

M. DWYER AND N. C. STICKLAND

partment of Veterinary Basic Sciences, The Royal Veterinary College, London, UK

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STRACT

ıtritional restriction during muscle fibre number development invariably has a more detrimental effect on biceps brachii than on the soleus. The difference may be due to the relative proportions of fibre types in muscles or the anatomical location of the muscles. To distinguish between the effects of location and re type the biceps (fast, cranial and proximal), soleus (slow, caudal and distal), and extensor digitorum ıgus (EDL) (fast, caudal and distal) were examined from control and undernourished guinea pig neonates. 40% reduction in maternal intake resulted in a reduction in neonate body and muscle weights ($P <$ 05), biceps ($P < 0.05$) and EDL fibre numbers ($P < 0.005$), but did not affect soleus fibre number. At th the ratio of fast fibres to slow was 7.5 for the biceps, 7.0 for the EDL, and 1.5 for the soleus. The ect of undernutrition on muscle fibre number therefore seems to be a function of the fibre types in that scle.

TRODUCTION

the guinea pig, a 40% reduction in maternal feed ake has been shown to cause a decrease in muscle re numbers in the biceps brachii but not in the eus of the progeny (Ward & Stickland, 1991). It ms that developing muscles with a relatively high w-twitch content invariably suffer a smaller deficit muscle fibre number than more fast-twitch muscles h maternal undernutrition (Howells et al. 1977; di et al. 1982). This suggests that the effect of trition on muscle fibre development may be a ction of the relative proportions of the fibre types. wever, an alternative explanation may be the atomical location of the muscles in the body. mmalian development proceeds in a cephalocaudal d proximodistal direction with the head being most vanced, the trunk in advance of the limbs, and elimbs in advance of the hindlimbs (Fleagle et al. 5). In addition, a proximodistal gradient can be n within the limbs. It is possible that, being a less ture muscle than the biceps, nutrients are preferenly directed towards the soleus such that muscle re numbers are maintained.

In order to determine whether muscle fibre number reduction is a function of fibre types or due to anatomical location, a third muscle, the EDL, was investigated. This muscle is located in the distal hind limb in close proximity to the soleus, in contrast to the biceps brachii in the proximal fore limb. The EDL is also involved in propulsive actions and hence is a predominantly fast muscle like the biceps brachii. Examination of this muscle should allow a differentiation to be made between the effects of muscle fibre types and anatomical location. This investigation was designed to test the hypothesis that the anatomical location of a muscle may be as important as the proportion of muscle fibre types (and thereby the proportions of secondary and primary fibres during development) in influencing the effect of prenatal undernutrition on muscle fibre number determination.

MATERIALS AND METHODS

This project involved 6 litters of neonate Dunkin–Hartley guinea pigs. Guinea pig dams were mated postpartum in a harem and assigned to a nutritional treatment on d 2 of gestation. The dams of 3 litters

respondence to Dr C. M. Dwyer, Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London 1 0TU, UK.

Table. *Comparison of gross body weights and lengths for control and restricted neonate guinea pigs*

Parameter	Control	40% restricted	% difference	*P*
Number	12	13		
Age (d)	67.3 ± 1.20	66.3 ± 1.33		n.s.
Body wt (g)	84.89 ± 6.12	55.71 ± 5.53	34.37	< 0.005
Liver wt (g)	4.646 ± 0.447	2.946 ± 0.447	36.59	< 0.05
Biceps wt (mg)	57.72 ± 4.49	37.18 ± 4.58	35.59	< 0.005
Soleus wt (mg)	26.10 ± 1.52	16.44 ± 2.03	37.01	< 0.005
EDL wt (mg)	25.13 ± 1.97	16.19 ± 1.99	35.58	< 0.005
CR length (cm)	11.02 ± 0.16	9.16 ± 0.30	16.88	< 0.001
Tibial length (mm)	27.23 ± 0.46	23.56 ± 0.75	13.48	< 0.001
Humeral length (mm)	21.53 ± 0.36	19.03 ± 0.51	11.61	< 0.001

were fed a standard diet at ad lib levels throughout gestation and formed the control group. The other 3 were pair fed to weight matched controls at a 40% restricted feed level.

Guinea pigs littered at a mean gestational age of 66.83 ± 0.76 d. Mean litter sizes were 4.00 and 4.33 for control and restricted litters respectively. All neonates (n = 25) were killed by an intraperitoneal injection (1 ml/kg body wt) of pentobarbitone sodium (Euthesate). Body weight, liver weight, crown–rump length and the lengths of the humerus and tibia were recorded. The biceps brachii, soleus and extensor digitorum longus (EDL) muscles were removed, weighed, and rapidly frozen. Whole muscle transverse sections (10 μm) were cut and stained to demonstrate ATPase activity using a modification of the technique of Guth & Samaha (1970) at pH 10.3 and 4.6. Total fibre number counts were made for each muscle and animal using a Seescan image analyser (Seescan, Cambridge, UK) and the ratio of fast to slow fibres was determined. For the purposes of this study this was taken to be the mean ratio of fibres showing high myosin ATPase activity after preincubation at pH 10.3 to those showing little or no staining (referred to as the ratio of positive to negative basic ATPase activity). Differences between control and 40% restricted animals were determined by unpaired t tests.

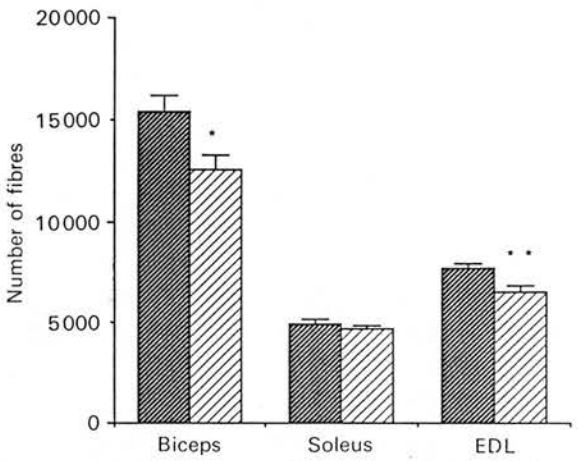

Fig. 1. Mean and S.E.M. of total fibre numbers for biceps, soleus and EDL from control (▩) and 40% restricted (▨) guinea pig neonates.

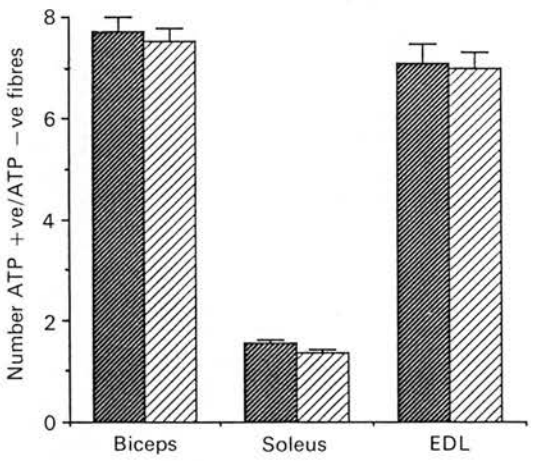

Fig. 2. Mean ratio of basic ATPase positive to basic ATPase negative fibres (fast- to slow-twitch fibres) for biceps, soleus and EDL from control (▩) and 40% restricted (▨) neonates, Bars, S.E.M.

RESULTS

Body, liver and muscle weights and body and bone lengths are given in the Table. For all weight and length parameters measured, the control group was significantly larger than the restricted group, demonstrating the effects of the 40% reduction in maternal food intake throughout gestation. Body weight, liver weight and muscle weights were all reduced by approximately one third in the restricted group. Crown–rump, tibial and humeral lengths were reduced by 16.9, 13.5 and 11.6%, respectively.

Muscle fibre number data are shown in Figure 1. Biceps and EDL fibre numbers were significantly reduced in the restricted group by 18.6% ($P < 0.05$) and 15.4% ($P < 0.005$), respectively. The soleus fibre

umber showed a small decrease of 5.4% in the estricted group; this was not significant.

The ratio of fast to slow fibres (basic ATPase ositive to negative) is shown in Figure 2. The soleus as a predominantly slow muscle with only 1.5 fast bres per slow fibre. The biceps and EDL were both ixed muscles of a predominantly fast nature. The tio of fast fibres to slow was approximately 7.5 in e biceps and 7.0 in the EDL (Fig. 2).

ISCUSSION

hese results demonstrate the significant reduction in ody and organ weights of neonates caused by a 40% striction of maternal dietary intake throughout estation. The weight of the biceps, soleus and EDL ere all reduced by equal amounts (approximately ne third). This was despite the known sparing of the oleus from the effects of undernutrition on muscle bre number (Ward & Stickland, 1991). The location the soleus and EDL, caudal and distal in relation to e biceps, did not appear to have any influence on the fect of undernutrition on muscle weight.

The most important results of this study was the nding that muscle fibre numbers in the biceps and DL of restricted neonates were reduced but that uscle fibre number in the soleus was not significantly fected. This suggests, therefore, that the influence of uscle location in the body was not important in termining the effect of undernutrition on muscle bre number. The percentage reduction in the EDL is ghtly less than in the biceps (respectively, 15.4 and .6%) which suggests that anatomical location may ay a small role. The biceps brachii and EDL, as fast pe muscles, contained 7–7.5 fibres per slow bundle, hereas the soleus contained only 1.5. It seems, erefore, that the proportion of fibre types in a uscle is the most important determinant of the fluence of nutritional restriction on total fibre mber.

The disproportionate effect of nutrition on fast uscles, in comparison with slow muscles, seems to be e to the relationship between twitch speed of a fibre d its generative origin, i.e. slow-twitch fibres tend to primary fibres in origin (Kelly & Rubenstein, 86). This is true of the guinea pig soleus where the uscle is predominantly slow and primary fibres count for about 33% of the total fibre number Vard & Stickland, 1991). This is in comparison with e biceps which is predominantly a fast-twitch muscle d where primary fibres account for less than 10% of fibres (Ward & Stickland, 1991). Primary fibre mbers have been shown to be unaffected by maternal undernutrition (Wigmore & Stickland, 1983; Handel & Stickland, 1987; Wilson et al. 1988; Ward & Stickland, 1991) or by denervation (McLennan, 1983; Ross et al. 1987; Harris et al. 1989). All these studies, however, showed that the secondary fibre population was susceptible to maternal undernutrition or to denervation during muscle development, leading to a reduced secondary fibre number. Slow muscles, being composed of a relatively high proportion of primary fibres, are therefore less susceptible to environmental influences in terms of reduced secondary fibre numbers.

A more severe nutritional regime, e.g. 50% of the ad lib quantity in the guinea pig throughout gestation (Ward, 1989), 30% throughout gestation in the rat (Wilson et al. 1988), or 50% through gestation and lactation in the rat (Bedi et al. 1982), does cause a reduction in fibre number in the soleus. This reduction was, however, to a lesser degree than the fibre number reduction in fast muscles. The effect of nutrition on the 2 types of muscle seen in this study was not absolute, therefore, but reflected the lesser effect of nutrition on slow muscles.

In conclusion, this study has demonstrated that the effect of nutrition on muscle fibre number is a function of the fibre types in that muscle. Fast muscles suffer a disproportionate reduction in fibre number with undernutrition due to their relatively high contribution to total fibre number made by the secondary fibre population. Slow muscles, with a greater proportion of primary fibres, are less affected by undernutrition.

ACKNOWLEDGEMENT

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REFERENCES

Bedi KS, Birzgalis AR, Mahon M, Smart JL, Wareham AC (1982) Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and refed adult animals. *British Journal of Nutrition* **47**, 417–430.

Fleagle JG, Samonds KW, Hegsted DM (1975) Physical growth of cebus monkey, Cebus albifrons, during protein and calorie deficiency. *American Journal of Clinical Nutrition* **28**, 246–253.

Guth L, Samaha FJ (170) Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28**, 365–367.

Handel SE, Stickland NC (1987) Muscle cellularity and birthweight. *Animal Production* **44**, 311–317.

Harris AJ, Fitzsimmons RB, McEwan JC (1989) Neural control of the sequence of myosin heavy chain isoforms in foetal mammalian muscles. *Development* **107**, 751–769.

Howells KF, Jordan TC, Piggott SM (1977) Effects of pre- and perinatal restricted food intake on the rat cerebellum. *Journal of Physiology* **272**, 6P.

KELLY AM, RUBENSTEIN NA (1986) Muscle histogenesis and muscle diversity. In *Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology*, vol. 29 (ed. C. Emerson, D. Fischman, B. Nadal-Ginard & M. A. Q. Siddiqui), pp. 77–84. New York: Alan R. Liss.

MCLENNAN IS (1983) Neural dependence and independence of myotube production in chicken hindlimb muscles. *Developmental Biology* **98**, 287–294.

ROSS JJ, DUXSON MJ, HARRIS AJ (1987) Neural determination of muscle fibre numbers in embryonic rat lumbrical muscles. *Development* **100**, 395–409.

WARD SS (1989) *The Effect of Undernutrition on Skeletal Muscle Development and Growth in the Guinea Pig*. PhD thesis, University of London.

WARD SS, STICKLAND NC (1991) Why are slow and fast muscles differentially affected during prenatal undernutrition? *Muscle and Nerve* **14**, 259–267.

WIGMORE PMC, STICKLAND NC (1983) Muscle development in large and small pig foetuses. *Journal of Anatomy* **137**, 235–245.

WILSON SJ, ROSS JJ, HARRIS AJ (1988) A critical period for formation by prenatal undernutrition in rats. *Development* **102**, 815–821.

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he effect of maternal undernutrition on the growth nd development of the guinea pig placenta

M. Dwyer, A.J.A. Madgwick, A.R. Crook & N.C. Stickland
artment of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU

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stract

al growth is known to be correlated with the size of placenta and the exchange surface area. Reduction the growth of the materno-fetal exchange surface as may be a mechanism by which the effects of ternal undernutrition on fetal growth are medi-d. In the compact placenta of the guinea pig the hange surface is equivalent to the peripheral laby-th. The effect of a 40% reduction in maternal feed ake on the growth of the peripheral labyrinth was estigated in pregnant guinea pigs between gesta-nal days 25 and 65. Fetal and placental weights re significantly reduced in the last trimester by 32% l 38% respectively ($P<0.01$). Placental efficiency in ly gestation was significantly impaired in re-cted animals but equivalent to *ad lib.* fed controls the last trimester. The volume of the peripheral yrinth increased as a percentage of the total placen-volume with gestational age. Restricted placentae ded to be composed of a smaller volume of periph-l labyrinth tissue in early gestation. It is suggested t maternal undernutrition results in an impaired delayed expansion of the peripheral labyrinth in ly gestation causing a reduction in placental effi-ncy. By the last trimester the weight of the ipheral labyrinth of restricted animals was reduced 33% ($P<0.05$). The weight of the peripheral laby-th was also significantly correlated with fetal ght during the last trimester, suggesting that fetal ght is limited by the size of the peripheral labyrinth he later stages of gestation.

roduction

ose linear relationship between fetal and placental ghts has been demonstrated in many species (eg. nea pig: Saintonge & Rosso, 1981; Garris, 1983; es & Parer, 1983; pig: Wootton, McFadyean & per, 1977; Wigmore & Stickland, 1985; rat: Rosso, 0; sheep: Owens, Allotta, Falconer & Robinson, 5; rabbit: Bruce & Abdul-Karim, 1973). Furthermore several studies, in the guinea pig, rabbit, sheep and pig, indicate that factors influencing growth affect the placenta before fetal growth is compromised (Barr, Jensh & Brent, 1970; Bruce & Abdul-Karim, 1973; Anderson, 1975; Mellor, 1983; Jansson, Thordstein & Kjellmer, 1986; Madgwick, 1991). This suggests that the growth of the placenta is a major determinant of fetal growth rate.

In the pregnant guinea pig a strenuous exercise programme led to a reduction in fetal and placental weights (Nelson, Gilbert & Longo, 1983). This was found to be associated with a reduced placental diffusing capacity and with a reduction in the total placental surface area (Smith, Gilbert, Lammers & Longo, 1983). In the diffuse porcine placenta, fetal weight also correlates well with placental area (Wigmore & Stickland, 1985). Additionally, in humans, small-for-gestational-age infants were found to be associated with a reduced villous surface area of the placenta (Woods, Malan & Heese, 1982). These results suggest that the growth of the materno-fetal exchange surface area may be an important aspect of placental growth influencing fetal development.

The guinea pig placenta is a compact, haemomonochorial disc where only one layer, the syncytiotrophoblast, seperates the maternal and fetal circulations (reviewed by Steven, 1975). The developed placenta is composed of two major components, the peripheral labyrinth and the interlobium, which are morphologically distinct (Kaufman & Davidoff, 1977). In the peripheral labyrinth fetal and maternal lacunae are closely apposed and, hence, this structure functions as the main exchange area of the placenta. The interlobar syncytial region of the placenta is characterised by an absence of fetal vessels and seems to be primarily associated with protein metabolism and resorption from the maternal blood flow (Davidoff, 1973). As gestation progresses the relative volume of the interlobium declines and of the peripheral labyrinth increases (Kaufman & Davidoff, 1977; personal observation). If the materno-fetal exchange surface area is an important determinant of fetal growth it would be expected that fetal growth would be closely associated with the growth of the peripheral labyrinth. In this study the effects of a 40% reduction in maternal feed intake on the growth of the placental components was

respondence: C. Dwyer, Veterinary Basic Sciences, The Royal erinary College, Royal College Street, London, NW1 0TU.

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Fig. 1. Sagittal section of part of the placental disc at 35 days gestation stained to demonstrate alkaline phosphatase. The major placental components are: IL interlobium, PL peripheral labyrinth, CL central labyrinth.

investigated. This level of undernutrition has previously been shown to cause a 35–40% reduction in fetal body weight by term (Ward & Stickland, 1991). The hypothesis tested in this investigation was that undernutrition would reduce the normal elaboration of the peripheral labyrinth resulting in impaired fetal growth.

Materials and methods

In this study guinea pigs of the Dunkin-Hartley strain were used. Guinea pigs were postpartum mated in a harem, taking the day of littering as day 0 of the subsequent pregnancy, and weight-matched pairs assigned to a control (*ad libitum*) or restricted (60% of *ad libitum*) feeding regime. All animals were fed Short and Gammage SG1 pellets and allowed free access to drinking water supplemented with vitamin E (α-tocopherol acetate, Roche Products Ltd). Animals were not given access to hay to ensure accurate regulation of dietary intakes.

A control and restricted pair of animals was sacrificed at 5 day intervals from day 25 of gestation to day 65 (term=68 days). Fetuses and placentae were then excised, trimmed of non-placental membranes, and blotted dry. Fetal and placental weights were recorded. A sagittal section of approximately 5 mm thickness was cut along the major radial axis of the placenta and mounted in O.C.T. compound (BDH) on a cork chuck. The placentae were than rapidly frozen in Arcton 12 (ICI) cooled in liquid nitrogen. Cryostat sections of 10 μm thickness were cut at –25°C and air dried onto clean, glass slides for 1 hour. Different components of the guinea pig placenta have different enzyme functions (Kaufmann & Davidoff, 1977). Unfixed cryostat sections were therefore stained to demonstrate alkaline phosphatase (after Bancroft and Stevens, 1982), and counterstained with neutral red, to differentiate between placental components (Fig. 1). The placenta could be divided into three distinct regions by this method: the central labyrinth which stained strongly for alkaline phosphatase and was characterised by large vessels; the peripheral labyrinth which exhibited lighter staining and many small vessels of both fetal and maternal origin; and the interlobium which did not stain for alkaline phosphatase and contained only maternal lacunae. The transitional region between the interlobe and labyrinth was also characteristically stained strongly for alkaline phosphatase associated with intense carbohydrate metabolism in this region (Kaufman & Davidoff, 1977).

The relative volumes of each placental component were determined by direct measurements of areas using a Seescan Image Analysis machine (Seescan plc, Cambridge, UK). The border between the interlobe and labyrinth was defined by a marked change in staining pattern for alkaline phosphatase. The border between the periphery and centre of the labyrinth was taken to be where the mean luminal width of lacunae exceeded 40 μm, as defined by Kaufmann and Davidoff (1977).

The weight of each placental component was also termined for each placenta using the DeLesse prin-ple, that is :

$W_c / W_t = V_c / V_t$

here W_c is the component weight, W_t is the total acental weight and V_c / V_t is the component volume action. This was used to examine the relative growth each placental component with increasing gesta-nal age.

esults

acental and fetal weights

ne variations in placental weight with gestational e are shown in Fig. 2a. Control placentae showed an parent increase in weight throughout gestation. The stricted placentae appeared to have reached their aximum growth by day 45 and did not increase in ight thereafter. This resulted in an average deficit placental weight of 32.5% (P<0.01, paired t-test) in the restricted animals within the last trimester (days 50–65).

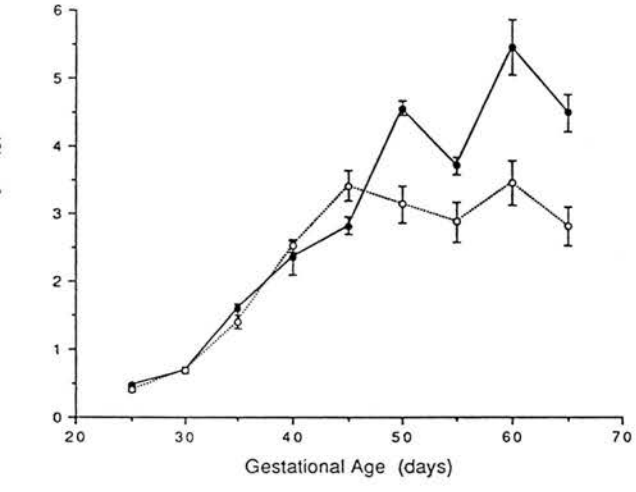
The change in fetal weight with gestational age is shown in figure 2b. Restricted animals were 38.5% (P<0.01, paired t-test) lighter than control fetuses in the last trimester.

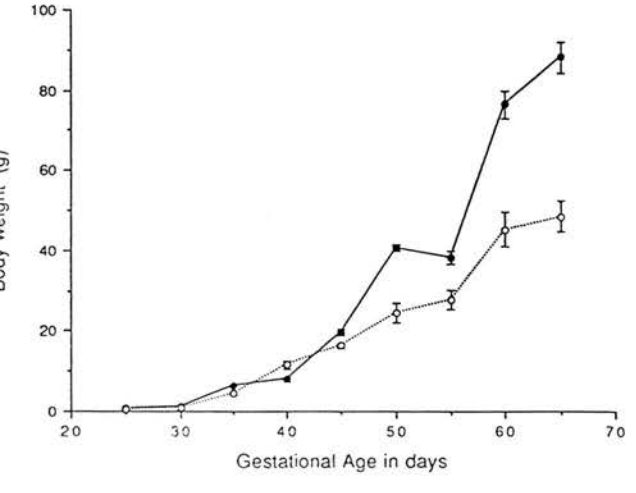
Placental efficiency

The feto-placental weight ratio was calculated for each animal as an indirect measurement of placental efficiency (Mellor, 1983). As gestation proceeded 1 g of placenta was able to support a greater mass of fetal tissue in both nutritional groups, reaching a maximum of nearly 20 g of fetus by 65 days gestation. The regression of log feto-placental ratio on gestational age was calculated for both groups (Fig. 3). Regression coefficients of 0.06 ± 0.002 and 0.07 ± 0.0024 were obtained for control and restricted groups respectively, and were significantly different (P< 0.01). Restricted placentae, therefore, appeared to have a significantly reduced efficiency in early gestation (day 25), but a similar efficiency to control placentae by the last trimester (Fig. 3).

Growth of placental components

The changes in relative volume of each placental component with gestational age are given in Fig. 4a and b. The relative volume of the interlobium declined with increasing gestational age in both nutritional groups, regression coefficients were negative and significantly different from zero (C: b=–0.0055 ± 0.0008; R: b=–0.0072 ± 0.0007, P<0.001). The relative volume of the peripheral labyrinth, however, increased with gestational age (C: b=0.0057 ± 0.0008; R: b=0.0069 ± 0.0007, P<0.001). There was no change in the relative volume of the central labyrinth with gestational age (Fig. 4b). When nutritional groups were compared, the difference in regression slopes tended towards significance for the change in interlobium weight on





. 2. Change in (a) placental weight and (b) fetal weight with tational age of control (●) and restricted (o) guinea pig fetuses. ues are litter means (± SE).

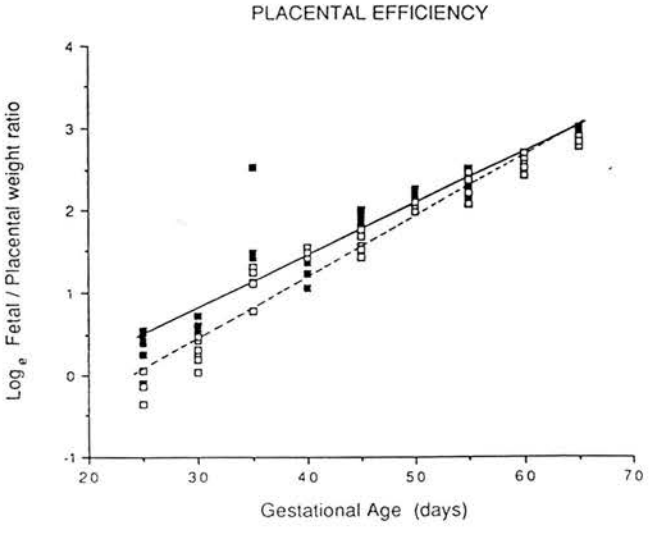
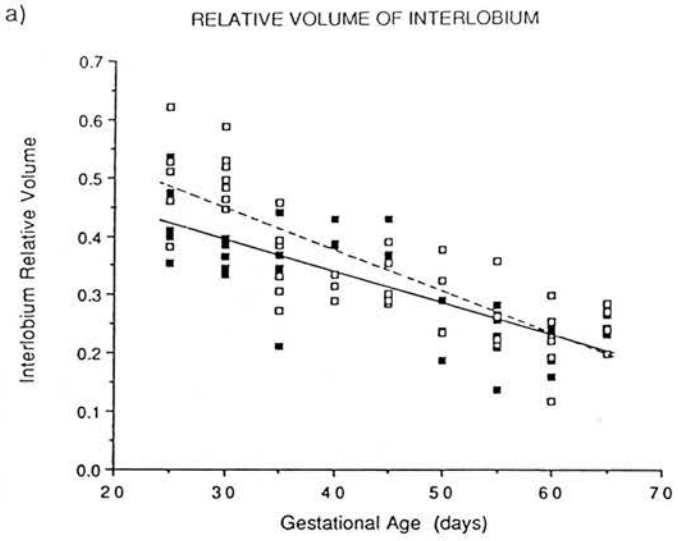


Fig. 3. Regression of log e feto-placental weight ratio against gestational age for control (■) and restricted (□) fetuses.



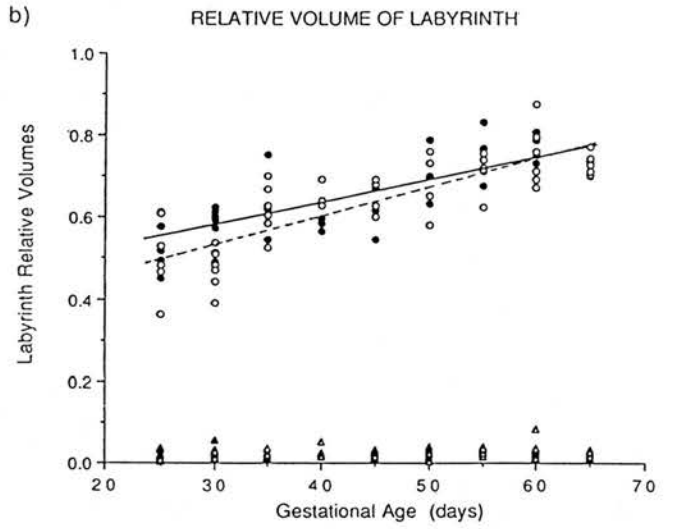
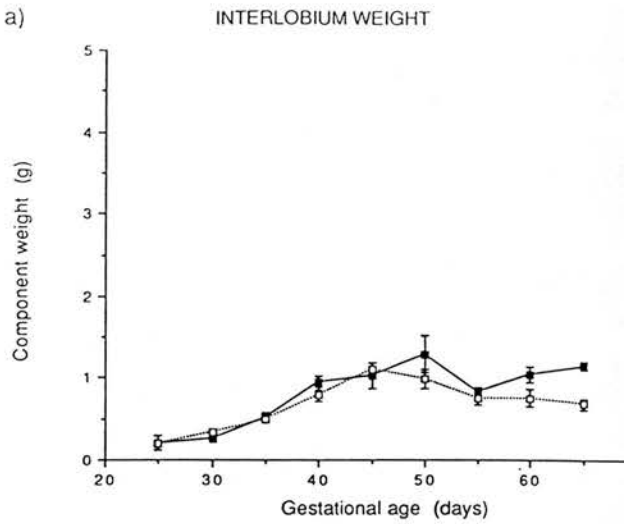


Fig. 4. Change in relative volume of (a) interlobium and (b) peripheral (Δ) and central (▲) labyrinths with gestational age for control (solid symbols and lines) and restricted (open symbols, broken lines) animals.



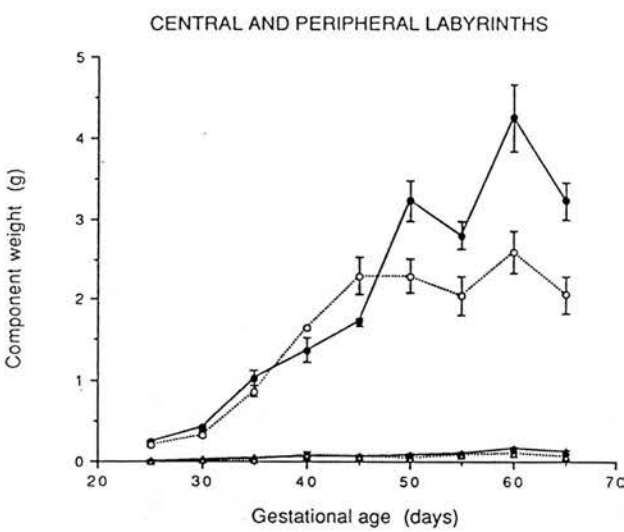


Fig. 5. Change in placental component weights with age, of th (a) interlobium, and (b) labyrinths, for control (solid symbols an lines) and restricted (open symbols, broken lines) fetuses.

gestational age (P=0.1), such that restricted placentae tended to contain relatively more interlobium and relatively less peripheral labyrinth tissue in early gestation.

The absolute weights of components were also calculated and are shown in Figure 5a and b. The weight of the interlobium increased similarly in both groups up to day 45 (Fig. 5a). Thereafter it appeared to decline in the restricted group and was maintained in the control group. Restricted animals had a 25.9% reduction in interlobium weight in the last trimester (P<0.05).

The change in the weight of the peripheral labyrinth tissue (Fig. 5b) followed a similar pattern to changes in total placental weight (Fig. 2a). That is, there was no increase in peripheral labyrinth weight in the restricted group after day 45 of gestation, whereas the weight of the peripheral labyrinth in the control grou continued to increase in weight with gestational age This resulted in a significant deficit of 33.4% (P<0.05 in restricted animals within the last trimester (day 50–65).

Relationships with fetal weight

The correlation of fetal weight with the growth of th placenta and of the peripheral labyrinths was investi gated. Similar relationships were noted in both contro and restricted groups, therefore nutritional group were combined (Table 1). Fetal weight was consist ently and significantly correlated with both placenta and peripheral labyrinth weight only after day 50 o gestation. Fetal weight was not correlated wit relative peripheral labyrinth volume at a given gesta tional age, except at 30 days gestation.

ble 1. The correlation of fetal weight with placenta weight, ght of the peripheral labyrinth (PL), and relative PL volume n gestational age.

ys)	Placenta weight	PL weight	PL volume
	r=0.63 P<0.05	r=0.55 P=0.079	NS
	NS	r=0.67 P<0.01	r=0.67 P<0.01
	NS	r=0.54 P=0.082	NS
	NS	NS	NS
	NS	NS	NS
	r=0.97 P<0.001	r=0.82 P<0.05	NS
	r=0.81 P<0.001	r=0.83 P<0.001	NS
	r=0.95, P<0.001	r=0.95 P<0.001	NS
	r=0.96, P<0.001	r=0.94 P<0.001	NS

ble 2. The correlation of placental efficiency with placenta ght, PL weight, and relative PL volume for the second (day 45) and third trimesters (day 50–65).

up	Placenta weight	PL weight	PL volume
trol 25–45 days	r=0.86 P<0.001	r=0.87 P<0.001	r=0.35 0.05<P<0.1
trol 50–65 days	NS	NS	NS
25–45 days	r=0.87 P<0.001	r=0.86 P<0.001	r=0.77 P<0.001
50–65 days	NS	NS	NS

ationships with placental efficiency

relationships between placental efficiency and cental weight, peripheral labyrinth weight and tive volume were investigated (Fig. 6a, b & c). en placental efficiency was low, ie. during early to gestation (Fig. 3), it was highly correlated with n placental and peripheral labyrinth weights. At a cental weight of greater than 2 g, or a peripheral rinth weight of 1.5 g, there was no correlation with cental efficiency (Fig. 6a & b). The plot of relative ume of peripheral labyrinth against placental effi- cy demonstrated that large changes in peripheral rinth volume were associated with only small nges in placental efficiency (Fig. 6c) when placental iency was low (mid-gestation). Additionally, a pe- heral labyrinth volume of 70% was associated with cental efficiencies of 5 to 20 g foetus per g placenta . 6c).

ince many changes in placental function appear to r in the last trimester the correlation of the par- eters with placental efficiency were investigated for mid trimester (days 25–45) and the last trimester s 50–65). These results are shown in Table 2. For n nutritional groups increased placental efficiency correlated with placental and peripheral laby- h growth in the second but not the third trimester.

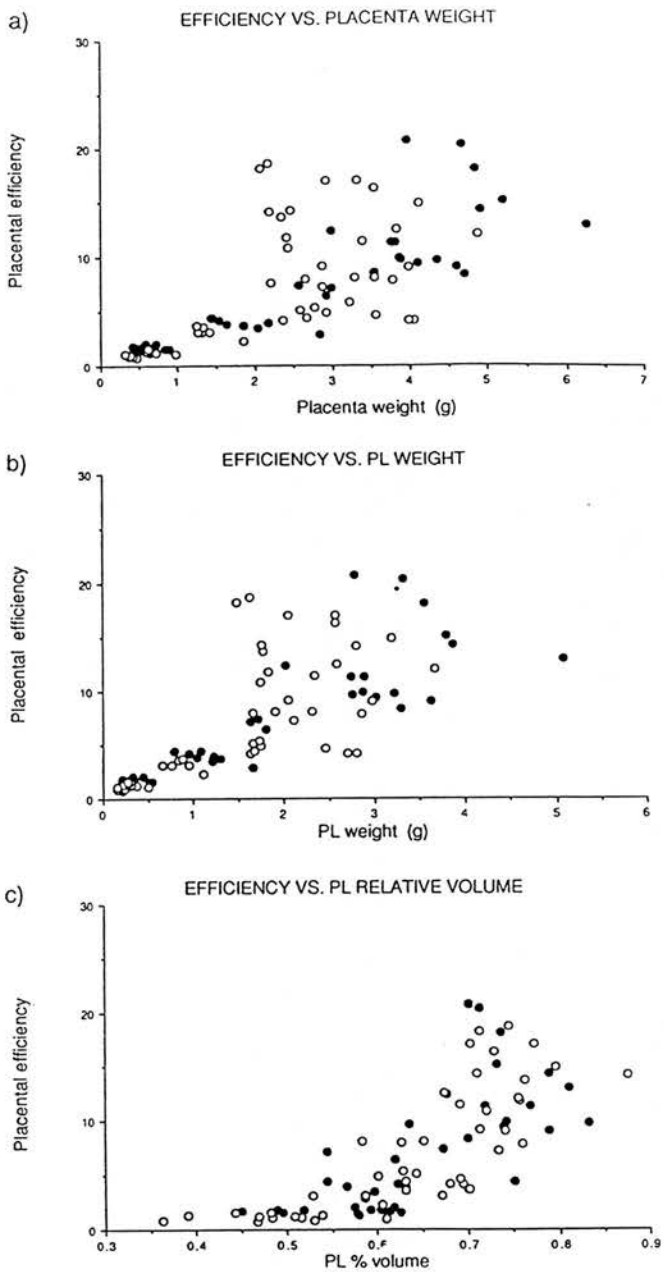


Fig. 6. Relationship between placental efficiency (feto-placental weight ratio) and (a) placenta weight, (b) peripheral labyrinth weight, and (c) relative peripheral labyrinth volume, for control (●) and restricted (o) fetuses.

Discussion

The main aim of this study was to investigate whether the reduction in fetal weight caused by maternal undernutrition could be attributed to restricted growth of the placental exchange surface areas. In addition, the relationship between the size of the exchange surface areas and placental efficiency, defined as weight of fetus supported per gramme of placenta (Mellor, 1983), was examined.

The major period of placental growth in the guinea pig is between days 25 and 45 of gestation (Fig. 2a; Garris, 1983; Bjellin, Sjöquist & Carter, 1975). Growth rate of the placenta slows or stops over the last trimester. Maternal undernutrition, strenuous exercise or

rine artery ligation all cause a reduction in placen-
weight in the guinea pig (Nelson *et al*, 1983; Jones
arer, 1983; Jansson *et al*, 1986; Carter & Detmer,
0; Madgwick, 1991). Reduced placental weights are
ociated with reduced placental hyperplasia and
ertrophy (Rosso, 1980; Hastings-Roberts & Zeman,
7).

he guinea pig placenta becomes increasingly effi-
t as gestation progresses (Fig. 3) even after the
enta has ceased to increase in weight. The effi-
cy of placentae of restricted animals was impaired
he second trimester, but was similar to control
entae by the third trimester. Total placental blood
in the guinea pig and rabbit increases with ad-
cing gestation (Bruce & Abdul-Karim, 1973;
lin *et al.*, 1975; Peeters, Sparks, Grutters, Girard
attaglia, 1982). In addition, in human placentae,
placental blood flow and ventricular output in-
se with gestational age (Sutton, Plappert &
bilet, 1991), such that placental blood flow is one
d of maternal cardiac output by the last trimester.
increase in placental blood flow per g of placental
ue, however, does not occur until the last trimester
ne guinea pig (Peeters *et al.*, 1982). Therefore this
nge could only account for the increased efficiency
lacental transport seen in both nutritional groups
ng the last trimester.

he placenta shows morphological adaptations with
ational age which increase its efficiency. During
last trimester in the guinea pig the size of the fetal
els increase, as does the surface area of maternal
els by proliferation of microvilli (Firth & Farr,
7). There is also an expansion in the materno-fetal
nange surface area with gestational age in the
ea pig as shown by the increase in relative volume
e peripheral labyrinth with gestational age (Kauf-
n & Davidoff, 1977; Fig. 4b). The relative
portion of interlobial volume declines from 100% at
12 (Kaufmann & Davidoff, 1977) to about 20% by
65 (Fig. 4a). There is a corresponding increase in
relative volume of the peripheral labyrinth from
at day 12 (Kaufmann & Davidoff, 1977) to 75% by
65 (Fig. 4b). The large vessels of the central laby-
h occupy about 2% of the total placental volume
ughout gestation. The peripheral labyrinth of the
ricted placentae tended to account for a greater
portion of the placental volume, and the peripheral
rinth proportionally less volume, than in control
entae in early gestation (Fig. 4). This may be due
etarded development of the restricted placentae,
that they were less mature at an equivalent age.
ental efficiency was correlated with placental
ght, peripheral labyrinth weight and relative pe-
eral labyrinth volume before day 50 only (Table 2).
refore, during the second trimester, expansion and
vth of the exchange surfaces were responsible for
eased placental efficiency, even though they were
related to fetal weight gain during this time
le 1).

Uterine blood flow, in the guinea pig, increases between gestational days 5 and 20, peaking at day 15 (Garris, 1984a). This is thought to be associated with the early establishment and development of the feto-placental unit (Garris, 1984a) and with the observed increase in uterine weight up to day 15 (Garris, 1984b). Dietary restriction, however, causes a reduction in blood flow to the placenta and uterus (Rosso & Kava, 1980; Ahokas, Anderson & Lipshitz, 1983) and a reduction in the normal expansion of maternal cardiac output with pregnancy (Ahokas *et al.*, 1983). This suggests that the impaired early placental development and efficiency of restricted placentae may have been caused by a reduced uterine blood flow which affected the exchange surface areas.

The weights of all placental components increased with gestational age as the weight of the total placenta increased (Fig. 5). The growth of the peripheral labyrinth followed a similar pattern to the growth of the placenta, reaching a plateau at day 45–50 (Fig. 5b). Smith *et al.* (1983) demonstrated in exercised guinea pigs that fetal weight is correlated with placental diffusing capacity and hence with both maternal and fetal exchange areas. In the guinea pig the main exchange area of the placenta is the peripheral labyrinth (Davidoff, 1973), therefore the correlation of fetal weight with placental weight and the relative volume and weight of the peripheral labyrinth was investigated (Table 1). Fetal weight was consistently correlated with placental and peripheral labyrinth weights only after day 50. This time point corresponds to an increase in fetal growth rate (Fig. 2b), a decrease in placental growth rate (Fig. 2a; Bjellin *et al.*, 1975; Garris, 1984b) and a second peak in uterine blood flow (Garris, 1984a). Other studies in the guinea pig and rabbit have shown that there is no correlation between fetal weight and placental blood flow until the last trimester (Bruce & Abdul-Karim, 1973; Peeters *et al.*, 1982; Jansson *et al.*, 1986; Jansson & Persson, 1990). In addition a linear correlation of placental blood flow and oxygen usage with gestational age is seen only after day 50 (Block, Johnson, Sparks & Battaglia, 1988). Since placental blood flow is correlated with placental weight (Wootton *et al.*, 1977; Saintonge & Rosso, 1981; Garris, 1983; Carter & Detmer, 1990), it seems that a small placenta becomes growth-limiting for the fetus in the last trimester due to a reduced capacity for placental blood flow. Fetal growth rate is known to be limited by placental blood flow rate in late gestation (Jansson *et al.*, 1986). In the second trimester (days 25–45) placental weight does not appear to be a major factor limiting fetal growth. The relative weight of the exchange area also appears to be an important determinant of fetal growth only in the last trimester.

The results of Smith *et al.* (1983) were from adequately nourished animals, therefore the concentration of substrates in the maternal plasma would not have been a factor limiting fetal growth in their study. In the

esent study, however, the supply of nutrients over the acenta would be an important determinant of fetal owth. The supply of amino acids to the fetus is mainly fected by placental blood flow (Lasuncion, Lorenzo, lacin & Herrera, 1987; Jansson & Persson, 1990). e transfer of glucose is related predominantly to the ncentration in the maternal plasma (Lasuncion *et al.*, 87), up to a maximum defined by the maternal blood w (Krauer, Joyce & Young, 1973). In fetuses of 50 ys or older fetal growth is rapid (Fig. 2b) with a high quirement for amino acids (reviewed by Jones, 1976), erefore the placental perfusion rate and area of the exange surface are likely to be the most important terminants of fetal growth, as was the case.

In conclusion, the early growth of the peripheral oyrinth appeared to be compromised by maternal dernutrition (Fig. 4b) causing a reduced placental iciency (Fig. 3). This may have been caused by a duced uterine blood flow resulting in delayed matur- on of the placentae from restricted dams. During the cond trimester (days 25–45) placental efficiency was rrelated with the relative volume and weight of the acental exchange surface areas (Table 2). By the last mester, growth of the placenta and exchange areas s virtually complete (Figs. 2a & 4b), yet the effi- ncy of the placenta continued to increase (Fig. 3). is may have occurred by increased placental blood w per gramme of tissue, which is known to occur in e last trimester (Peeters *et al.*, 1982), and by a duction in the effective distance between maternal d fetal blood vessels (Firth & Farr, 1977). Although acental efficiency of the restricted placentae im- oved over the last trimester, fetal growth was paired by the smaller weight of peripheral labyrinth sue and hence the reduced exchange surface area.

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eferences

okas, R.A., Anderson, G.D. & Lipshitz, J. (1983) Effect of dietary estriction, during the last week only or throughout gestation, on ardiac output and uteroplacental blood flow in pregnant rats. *ournal of Nutrition*, **113**, 1766-1776.

derson, L.L. (1975) Embryonic and placental development during rolonged inanition in the pig. *American Journal of Physiology*, **29**, 1687-1694.

ncroft, J.D. & Stevens, A. (1982) *Theory and Practice of Histologi- al Techniques*, Churchill Livingstone.

rr, M., Jensh, R.P. & Brent, R.L. (1970) Prenatal growth in the lbino rat: Effects of number, intrauterine position and resorp- ions. *American Journal of Anatomy*, **128**, 413-428.

llin, L., Sjoquist, P-O.B. & Carter, A.M. (1975) Uterine, maternal lacental and ovarian blood flow throughout pregnancy in the uinea pig. *Zeitschrift für Geburtskunde und Perinatologie*, **179**, 79-187.

ck, S.M., Johnson, R.L., Sparks, J.W. & Battaglia, F.C. (1988) Uterine metabolism of the pregnant guinea pig as a function of estational age. *Pediatric Research*, **23**, 45-49.

ıce, N.W. & Abdul-Karim, R.W. (1973) Relationships between etal weight, placental weight, and maternal placental circulation n the rabbit at different stages of gestation. *Journal of Reproduc- ion and Fertility*, 32, 15-24.

Carter, A.M. & Detmer, A. (1990) Blood flow to the placenta and lower body in the growth-retarded guinea pig foetus. *Journal of Developmental Physiology*, **13**, 261-269.

Davidoff, M. (1973) Guinea pig placenta: Fine structure and development. *Acta Anatomica*, **86**, suppl 1, 23-46.

Firth, J.A. & Farr, A. (1977) Structural features and quantitative age-dependent changes in the intervascular barrier of the guinea pig haemochorial placenta. *Cell and Tissue Research*, **184**, 507-516.

Garris, D.R. (1983) Regional variations in guinea pig uterine blood flow during pregnancy: Relationship to intrauterine growth of the feto-placental unit. *Teratology*, **27**, 101-107.

Garris, D.R. (1984a) Uteroplacental vascular perfusion and blood flow during pregnancy in the guinea pig. *Biology of the Neonate*, **45**, 33-40.

Garris, D.R. (1984b) Intrauterine growth of the guinea pig feto-placental unit throughout pregnancy: Regulation by uteroplacental blood flow. *Teratology*, **29**, 93-99.

Hastings-Roberts, M.M. & Zeman, F.J. (1977) Effects of protein deficiency, pair-feeding, or diet supplementation on maternal, fetal, and placental growth in rats. *Journal of Nutrition*, **107**, 973-982.

Jansson, T., Thordstein, M. & Kjellmer, I. (1986) Placental bloodflow and foetal weight following uterine artery ligation. *Biology of the Neonate*, **19**, 172-180.

Jansson, T. & Persson, E. (1990) Placental transfer of glucose and amino acids in intrauterine growth retardation: Studies with substrate analogs in the awake guinea pig. *Pediatric Research*, **28**, 203-208.

Jones, C.T. (1976) Fetal metabolism and fetal growth. *Journal of Reproduction and Fertility*, **47**, 189-201.

Jones, C.T. & Parer, J.T. (1983) The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea pig. *Journal of Physiology*, **343**, 525-537.

Kaufmann, P. & Davidoff, M. (1977) The guinea pig placenta. *Ergebnisse der Anatomie und Entwicklungsgeschichte*, **53**, 5-91.

Krauer, F., Joyce, J. & Young, M. (1973) The influence of high maternal plasma glucose levels, and maternal blood flow on the placental transfer of glucose in the guinea pig. *Diabetologica*, **9**, 453-456.

Lasuncion, M.A., Lorenzo, J., Palacin, M. & Herrera, E. (1987) Maternal factors modulating nutrient transfer to the fetus. *Biology of the Neonate*, **51**, 86-93.

Madgwick, A.J.A. (1991) The effect of maternal undernutrition on foetal myogenesis and development in the guinea pig (*Cavia porcellus*). PhD. Thesis, University of London.

Mellor, D.J. (1983) Nutritional and placental determinants of foetal growth rate in sheep and consequences for the newborn lamb. *British Veterinary Journal*, **139**, 307-324.

Nelson, P.S., Gilbert, R.D. & Longo, L.D. (1983) Fetal growth and placental diffusing capacity in guinea pig following long-term maternal exercise. *Journal of Developmental Physiology*, **5**, 1-10.

Owens, J.A., Allotta, E., Falconer, J. & Robinson, J.S. (1985) Effect of restricted placental growth upon oxygen and glucose delivery to the foetus. In *Physiological Development of the Fetus and Newborn* (Jones, C.T. & Nathanielsz, P.W., eds.). pp 33-36. Academic Press, London.

Peeters, L.L.H., Sparks, J.W., Grutters, G., Girard, J. & Battaglia, F.C. (1982) Uteroplacental blood flow during pregnancy in chronically catheterised guinea pigs. *Pediatric Research*, **16**, 716-720.

Rosso, P. (1980) Placental growth, development and function in relation to maternal nutrition. *Federation Proceedings*, **39**, 250-254.

Rosso, P. & Kava, R. (1980) Effects of food restriction on cardiac output and blood flow to the uterus and placenta in the pregnant rat. *Journal of Nutrition*, **110**, 2350-2354.

Saintonge, J. & Rosso, P. (1981) Placental blood flow and transfer of nutrient analogs in large, average and small guinea pig littermates. *Pediatric Research*, **15**, 152-156.

Smith, A.D., Gilbert, R.D., Lammers, R.J. & Longo, L.D. (1983) Placental exchange area in guinea pigs following long-term maternal exercise: a stereological analysis. *Journal of Developmental Physiology*, **5**, 11-21.

Steven, D.H. (1975) Anatomy of the placental barrier. In *Comparative Placentation* (Steven, D.H., ed.), pp. 25-56. Academic Press, New York.

Sutton, M.G., Plappert, T. & Doubilet, P. (1991) Relationship between placental blood flow and combined ventricular output with

estational age in the normal human fetus. *Cardiovascular Research*, **25**, 603-608.
rd, S.S. & Stickland, N.C. (1991) Why are fast and slow muscles ifferentially affected during prenatal undernutrition? *Muscle nd Nerve*, **14**, 259-267.
gmore, P.M.C. & Stickland, N.C. (1985) Placental growth in the ig. *Anatomy and Embryology*, **173**, 263-268.
Woods, D.L., Malan, A.F. & Heese, H. de V. (1982) Placental size of small-for-gestational-age infants at term. *Early Human Development*, **7**, 11-15.
Wootton, R., McFadyean, I.R. & Cooper, J.E. (1977) Measurement of placental blood flow in the pig and its relationship to placental and foetal weight. *Biology of the Neonate*, **31**, 333-339.

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The effects of maternal undernutrition on maternal and fetal serum insulin-like growth factors, thyroid hormones and cortisol in the guinea pig

C.M. Dwyer & N.C. Stickland

Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU

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Abstract

The insulin-like growth factors (IGF-I and -II) are potential mediators of the effects of maternal undernutrition on fetal growth and muscle development. The effects of a 40% reduction in maternal feed intake on serum levels of the IGFs, the thyroid hormones and cortisol, were investigated for the last two trimesters (day 25 to birth). This level of undernutrition is known to cause a 35% reduction in fetal and placental weights, and a 20–25% reduction in muscle fibre number. Maternal IGF-I level was greater than non-pregnant levels on day 25 gestation, in both control and restricted dams, and declined with gestational age. The increase in IGF-I level in the 40% restricted group was approximately two-thirds that of control animals. Fetal serum IGF-I was also reduced in undernourished fetuses throughout gestation. Maternal IGF-II did not change with gestational age and was unaffected by undernutrition. Fetal IGF-II reached a peak at day 55 of gestation, this peak was greatly diminished by maternal feed restriction. Both IGF-I and IGF-II tended to be related to fetal, placental and muscle weights at day 65 of gestation.

Thyroid hormone concentration declined in maternal serum and increased in fetal serum with increasing gestational age. Levels were not significantly affected by undernutrition. Both triiodothyronine (T3) and thyroxine (T4) were correlated with IGF-I in maternal serum ($P<0.05$), but not in fetal serum. Cortisol levels were elevated by undernutrition in both maternal and fetal serum, and increased with gestational age. Cortisol was inversely correlated with serum IGF-I in both maternal and fetal serum.

Maternal serum IGF-I may mediate the effects of undernutrition on fetal growth by affecting the growth and establishment of the feto-placental unit in midgestation. Fetal IGF-I may mediate the effects on muscle growth, whereas IGF-II seems to be related to hepatic glycogen deposition. Cortisol may play a role via its effect on the IGFs, but the thyroid hormones are unlikely to be important until the late gestation/early postnatal period.

Correspondence to: C. Dwyer, Dept. of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU

Introduction

Restriction of maternal dietary intake to 60% of *ad libitum* intake throughout pregnancy results in a 35% reduction in fetal and placental weights and a 20–25% reduction in fibre number of fast muscles (Ward & Stickland, 1991; Dwyer & Stickland, 1992). Liver weight is also reduced by approximately one third, and body and bone lengths by 12–17% (Dwyer & Stickland, 1992). Uterine artery ligation in the rat and guinea pig, or maternal undernutrition of the rat and pig, results in preferential growth of the brain and heart at the expense of liver and muscle (Widdowson, 1971; Lafeber, Rolph & Jones, 1984; Ogata, Bussey, Labarbera & Finley, 1985; Fernandez, Menendez, Fernandez & Patterson, 1985). The insulin-sensitive tissues are, therefore, more affected by undernutrition than other organs.

The insulin-like growth factors (IGFs) may be mediators of the effects of undernutrition on muscle fibre number development. Fetal plasma levels of IGF-I are correlated with fetal body weight in late gestation (human: Gluckman & Brinsmead, 1976; Gluckman, Johnson-Barrett, Butler, Edgar & Gunn, 1983; Ashton, Zapf, Einschenk & MacKenzie, 1985; pig: Hausman, Campion & Buonomo, 1991; guinea pig : Jones, Lafeber, Rolph & Parer, 1990). IGF-II, however, has been reported to be both correlated (Bennett, Wilson, Liu, Nagashima, Rosenfeld & Hintz, 1983; Hausman *et al.*, 1991), and not correlated (Gluckman *et al.*, 1983; Ashton *et al.*, 1985; Lafeber, Jones & Price, 1987; Jones *et al.*, 1990), with birth weight. However, IGF-II does seem to be an important fetal growth regulator since an IGF-II-deficient mouse mutant yielded heterozygous progeny which were 40% smaller than the wild type (Dechiara, Efstratiadis & Robertson, 1990). This provides the first direct evidence of a physiological role for IGF-II in embryonic growth.

ra-uterine growth retardation (IUGR) by carun- my or artery ligation causes reduced tomedin activity measured by bioassay (Fal- , Forbes, Hart, Robinson & Thorburn, 1979; *et al.*, 1990). This is associated with reduced n IGF-I and elevated IGF-II (Lafeber, Price & , 1987; Jones, Gu, Harding, Price & Parer, 1988; *et al.*, 1990). Maternal fasting in the guinea pig, otein restriction in the rat, cause a depression of IGF-I and IGF-II (Pilistine, Moses & Munro, Jones *et al.*, 1990). However, fasting in the ant rat causes a decrease in fetal serum IGF-I oes not affect IGF-II (Davenport, D'Ercole & rwood, 1990a). Fetal levels of the IGFs are, there- ffected by maternal dietary manipulation.

F-II mRNA in fetal and neonatal rat muscle and s known to be depressed by glucocorticoids (Beck, ni, Senior, Byrne, Morgan, Gebhard & Bram- 1988; Levinovitz & Norstedt, 1989) and the d hormones have also been suggested as poten- egulators of IGF levels (Gaspard, Wondergem, mdzic & Klitgaard, 1978; Fagin, Fernandez- & Melmed, 1989; Ikeda, Fujiyama, Takeuchi, a, Mokuda, Tominga & Mashiba, 1989). Growth one is related to levels of IGF-I and growth in atal animals (eg. Eigenmann, Zanesco, Arnold & ch, 1984; and others). However, it does not seem an important factor in the maternal response to ancy (Merrimee, Zapf & Froesch, 1982) or in fetal h in the guinea pig (Jones, Rolph, Lafeber, Gu, ng & Parer, 1985). A short fast has been shown ct plasma IGF levels in the mid-gestation guinea ones *et al.*, 1990), however, a study of the effects onic maternal undernutrition on fetal IGF levels ing. The current study was designed to examine ffects of a 40% reduction in maternal dietary e, throughout gestation, on the plasma levels of GFs in both fetal and maternal serum. Cortisol and the levels of triiodothyronine (T3) and thy- (T4) were also measured in this study, due to potential role in influencing the IGFs. Growth one, however, was not considered to be a con- or to muscle development and was not measured s study.

rials and methods

roject involved 20 Dunkin-Hartley guinea pigs ing between 700 and 900 g at the start of the Guinea pigs were post-partum mated and ran- assigned to a nutritional regime two days after tion. Weight matched pairs of animals were fed Short & Gammage) pellets at control (*ad libitum*, els or pair-fed at 60% of *ad lib.* intake (R). ls were housed individually without hay and unlimited access to clean, fresh drinking water ning vitamin E (Roche products Ltd). A control stricted pair of animals was sacrificed at 5 day als from 25 days gestation until term. The time of 25 days was chosen as this is immediately before the appearance of primary myotubes (Ward & Stickland, 1991). Somite formation (41 pairs) is already completed approximately 5 days before the start of animal selection (Evans & Sack, 1973). Animals were killed by an overdose injection of sodium pentobarbitone (Euthesate), which also killed the fetuses. In a few cases older fetuses (days 60 and 65) were also given supplementary Euthesate. The fetuses were exposed by a midline abdominal incision. Blood samples were immediately collected from the mother and, where fetal size permitted, from the fetuses by cardiac puncture using heparanised syringes. Samples were left on ice for 1 hour, then spun in a centrifuge at 2000 g at 4°C for 15 min. Plasma samples were stored at –70°C until assayed. Fetal weights, and the weights of the biceps brachii and soleus muscles were recorded.

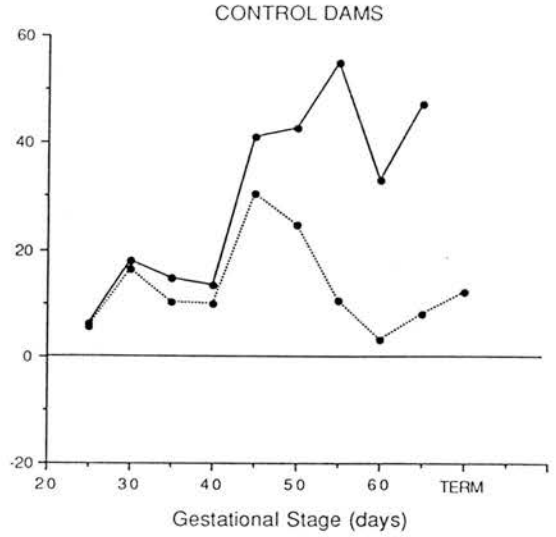
Prior to the IGF radioimmunoassays plasma samples were extracted with formic acid/acetone (Bowsher, Lee, Apathy, O'Brien, Ferguson & Henry, 1991) to remove binding proteins. Samples were measured against a serum standard obtained by serially diluting serum from healthy, young, male, guinea pigs. The assay procedures were modified after Buonomo, Grohs, Baile and Campion (1988) and Morrell, Dadi, More, Taylor, Dabestani, Buchanan, Holder and Preese (1989). The IGF-I antibody was a gift from the Institute of Child Health, and was a rabbit-antihuman polyclonal with a cross-reactivity with IGF-II of approximately 4% (Morrell *et al.*, 1989). The IGF-II antibody was a mouse anti-rat monoclonal (Sera-Lab) with a published cross-reactivity of 10% with IGF-I. However other researchers report a cross-reactivity for this antibody of less than 3% (Daughaday, Kapadia & Mariz, 1987). Cross-reactivity for each antibody was therefore assumed to be less than 5%. Briefly, 100 µl extracted sample, 200 µl assay buffer [0.03 M sodium phosphate, 0.01 M EDTA, containing 0.05% (v/v) Tween 20 and 0.02% (w/v) sodium azide, adjusted to pH 7.5] and 100 µl antibody were incubated at room temperature for 1 h. 100 µl 125I-IGF-I or IGF-II (Amersham) were added and samples were incubated at 4°C for 16–18 h. The bound fraction was precipitated with SAC-CEL second antibodies (IDS Ltd) and pellets were counted in a gamma counter.

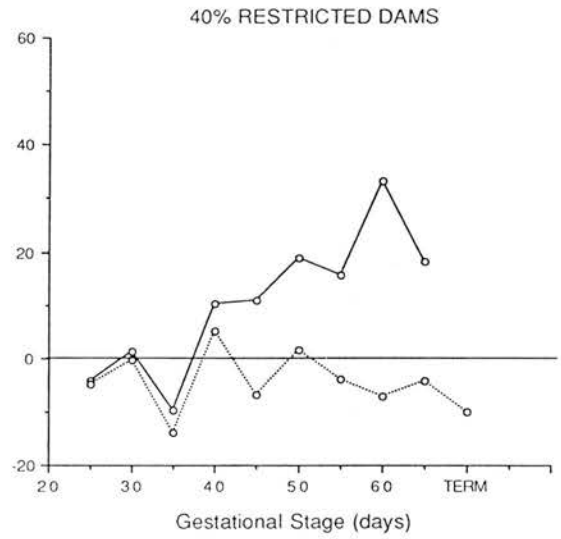
Cortisol, triiodothyronine (T3) and thyroxine (T4) were measured with specific radioimmunassays using kits and reagents supplied by IDS Ltd.

Results

Maternal weight gain

The net and overall changes in maternal weight are shown in Fig. 1, expressed as a percentage of weight at conception. Control animals showed a net weight gain of approximately 30% in the second trimester (days 25–45). In the restricted animals there was no increase in net weight. Net body weight gain declined in the last trimester in both nutritional groups. Control dams remained in a positive energy balance





1. Change in maternal weight as a percentage of conception nt for (a) control, and (b) 40% restricted guinea pig dams. For solid lines indicate overall weight change (ie. maternal plus ptus weights), and broken lines net weight change (ie. exclud- onceptus weight).

ughout gestation and had a net average gain of g per day. Restricted dams had a net loss of 0.80 g day.

l growth

regression of the square root of fetal body weight gestational age is shown in Fig. 2. Restricted ses grew significantly slower than control ani- s (regression slopes were significantly different, .001) resulting in a 38.5% deficit in the last ester (P<0.01, paired t-test). Regression coeffi- ts for the log (biceps brachii weight) and log eus weight) on log (gestational age) were signifi- ly different between nutritional groups for the ps (C: b=3.809 ± 0.191, R: b=2.867 ± 0.393, 01) but not for the soleus. The weights of both cles were significantly reduced in the restricted ses in the last trimester by approximately 45% .05).

Fig. 2. Regression of fetal body weight on gestational age for control (■) and restricted (□) fetuses.

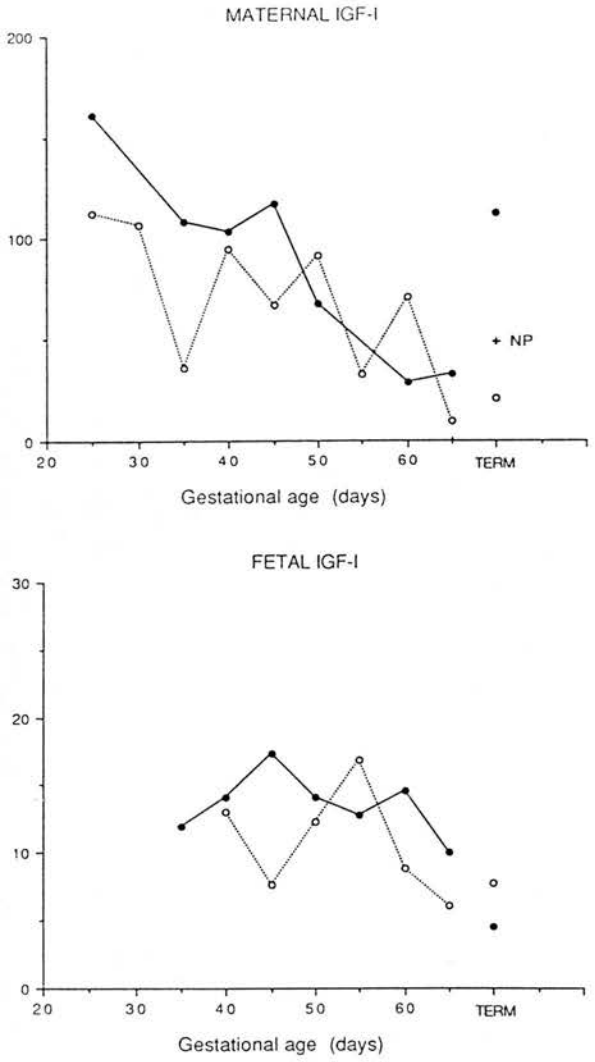
Serum IGF concentrations

Serum levels of IGF-I and -II are shown in Figs. 3 and 4. At day 25 of gestation control maternal IGF-I levels were approximately 3-fold, and restricted levels 2-fold, higher than IGF-I levels in non-pregnant serum (Fig. 3a). In both nutritional groups IGF-I levels declined between gestational days 25 and 65 (regression coefficients were significantly less than zero; C: $P<0.001$, R: $P<0.05$). By day 50 of gestation IGF-I concentrations were similar in both nutritional groups and in non-pregnant animals. When nutritional groups were compared, the difference between regression coefficients approached significance (C: $b= -3.274 \pm 0.457$, R: $b=-1.767 \pm 0.738$, $P=0.1$). The correlation of maternal serum IGF-I with litter weight is shown in Table 1. Maternal serum IGF-I was inversely related to mean fetal weight. In addition IGF-I was negatively correlated with litter weight and mean placental weight for all animals and in the control group. Maternal serum IGF-I was also correlated with maternal net weight gain or loss ($r=0.4569$, $P<0.06$).

Fetal serum was pooled for analysis at all ages except day 65 when sufficient plasma was obtained to permit analysis of individuals. Fetal levels of IGF-I were considerably lower than maternal levels (Fig. 3b). There was no obvious temporal variation with advancing gestation. Restricted levels tended to be lower than in controls, mean values to day 65 were 9.423 ± 1.314 and 12.761 ± 1.260 respectively ($P=0.085$, paired t-test). At day 65 IGF-I tended to be correlated with

Table 1. Correlation of maternal serum IGF-1 with fetal weight and placental weights for all animals and in nutritional groups.

Parameter	All	Control	Restricted
Litter weight	r=–0.687 P<0.005	r=–0.9318 P<0.005	r=–0.5049 NS
Mean fetus weight	r=–0.6933 P<0.001	r=–0.9406 P<0.001	r=–0.7610 P<0.005
Placental weight	r=–0.5362 P<0.01	r=–0.9652 P<0.001	r=–0.4323 NS



3. Change in IGF-I concentration of (a) maternal, and (b) fetal with gestational age, for control (●) and restricted (o) les. NP=non-pregnant female concentration.

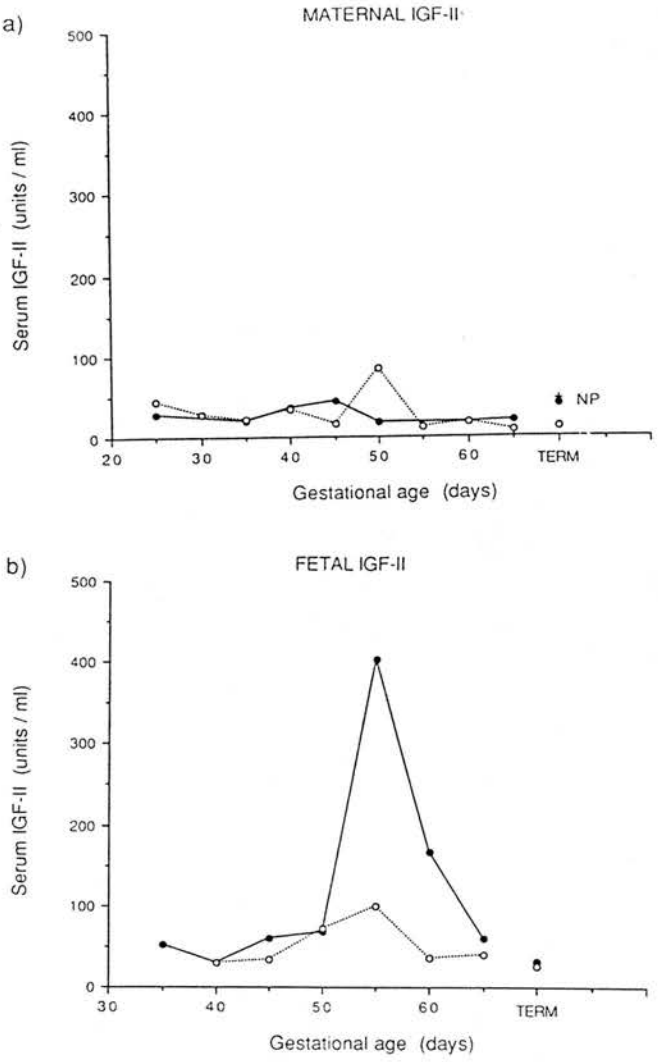


Fig. 4. Change in IGF-II concentration of (a) maternal, and (b) fetal sera with gestational age. Key as Fig. 3.

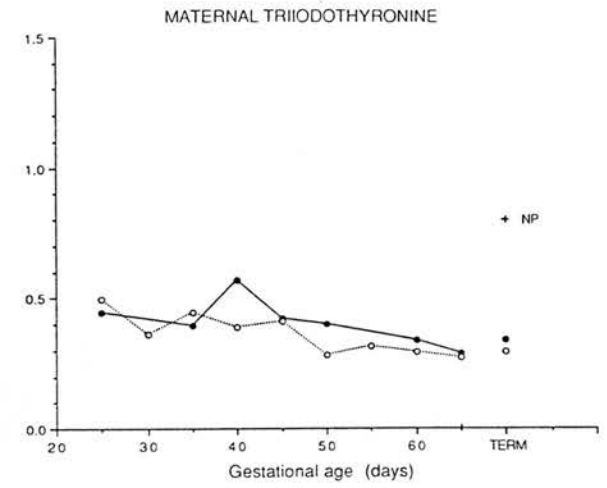
ental weight (r=0.6709, 0.05<P<0.1), fetal weight .5477, P=0.16) and the weights of the biceps bra- (r=0.5926, P=0.12) and soleus (r=0.5929, P=0.12). here were no significant effects of nutrition or nancy on maternal serum IGF-II concentrations; s were similar to one another, and to non-preg- levels, throughout gestation (Fig. 4a). There was rrelation between maternal IGF-II and total litter ht, fetal weight or placental weight. In addition, e was no correlation between maternal serum -II and changes in maternal weight with gestation. tal serum levels of IGF-II were only slightly er than maternal levels until day 55 gestation n there was a characteristic 8-fold increase in the rol animals (Fig. 4b). This peak was also seen in restricted animals but was markedly reduced, g only a 2-fold rise. The correlation of IGF-II with I levels and fetal parameters at 65 days of age was stigated. IGF-I levels were significantly correlated IGF-II levels (r=0.7402, P<0.05). IGF-II levels tended to be correlated with placenta weight 6681, 0.05<P<0.1), fetal weight (r=0.5922, P=0.12), and the weight of the biceps (r=0.5976, P=0.12) but not the weight of the soleus.

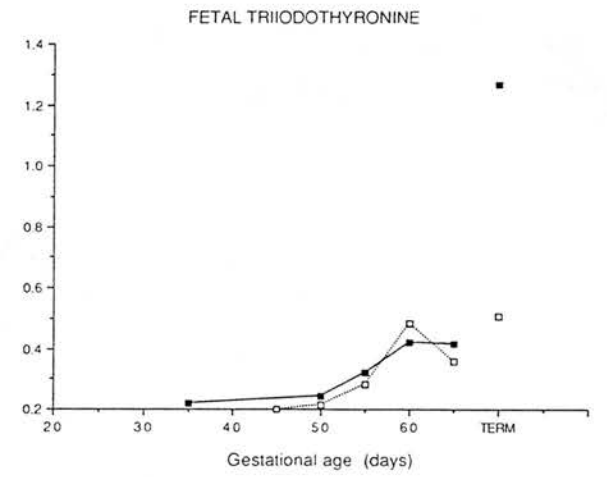
There was no relationship between maternal and fetal levels of IGF-I or IGF-II.

Thyroid hormones

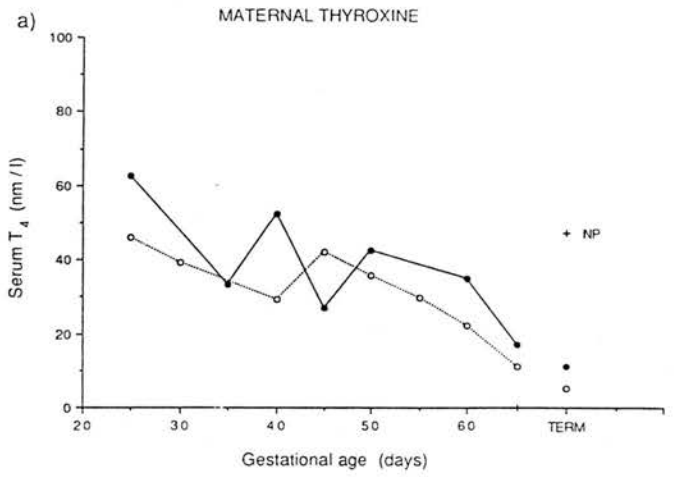
Serum levels of the thyroid hormones are shown in Fig. 5 and 6. Maternal T3 levels were considerably reduced in both control and restricted dams when compared to non-pregnant levels (Fig. 5a). The regression of T3 on gestational age was significantly less than zero for the restricted group (b=–0.00487, P<0.01) but not for controls. There were, however, no significant differences between nutritional groups when regression slopes were compared. Maternal levels of T4 were similar to non-pregnant levels at day 25 of gestation (Fig. 6a) and declined with increasing gestational age (C: b=–0.82 ± 0.33, 0.05<P<0.1; R: b=–0.68 ± 0.18, P<0.01). Both T3 and T4 were inversely correlated with increasing litter weight throughout gestation (r=–0.6913 and r=–0.7466 respectively, P<0.001).

Fetal levels of T3 and T4 were very low in mid-gestation (Fig. 5b and 6b) but increased rapidly from





5. Changes in T3 concentration of (a) maternal, and (b) fetal with gestational age. Key as Fig. 3.



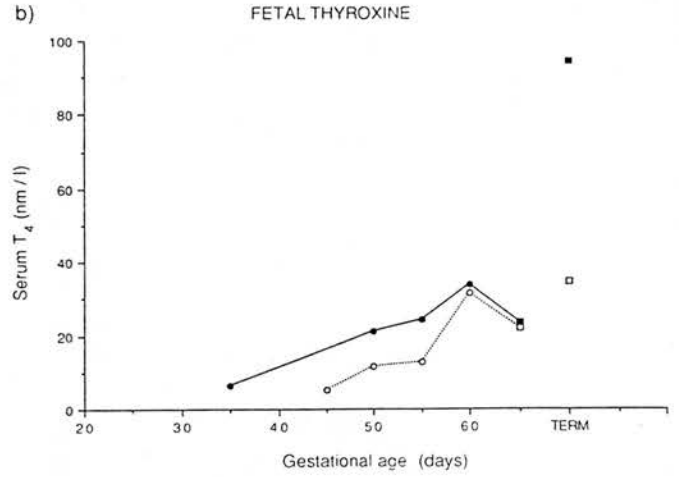


Fig. 6. Changes in T4 concentration of (a) maternal, and (b) fetal sera with gestational age. Key as Fig. 3.

50 onwards. Serum T3 and T4 increased signifi-
ly with gestational age in fetal serum (C: b=0.0076
0014, P<0.001 and b=0.58 ± 0.22, P<0.05; R: b=
91 ± 0.0036, P<0.05 and b=0.85 ± 0.40, 0.05<P<0.1
ectively). At term there was 3-fold increase in T3
T4 in control serum which was not seen in the
m from restricted neonates. The peak levels seen
rm exceeded concentrations seen in adult serum.
rum T3 and T4 were significantly correlated with
I in maternal serum (r=0.5488, P<0.05 and r=
06, P<0.005, respectively). However, there was no
elation between thyroid hormone levels and IGF-II
entrations in maternal serum, or between thyroid
nones and either IGF-I or IGF-II in fetal serum.
was related to fetal body weight at day 65
6758, P=0.096), but there was no relationship
een T4 and fetal weight.

isol

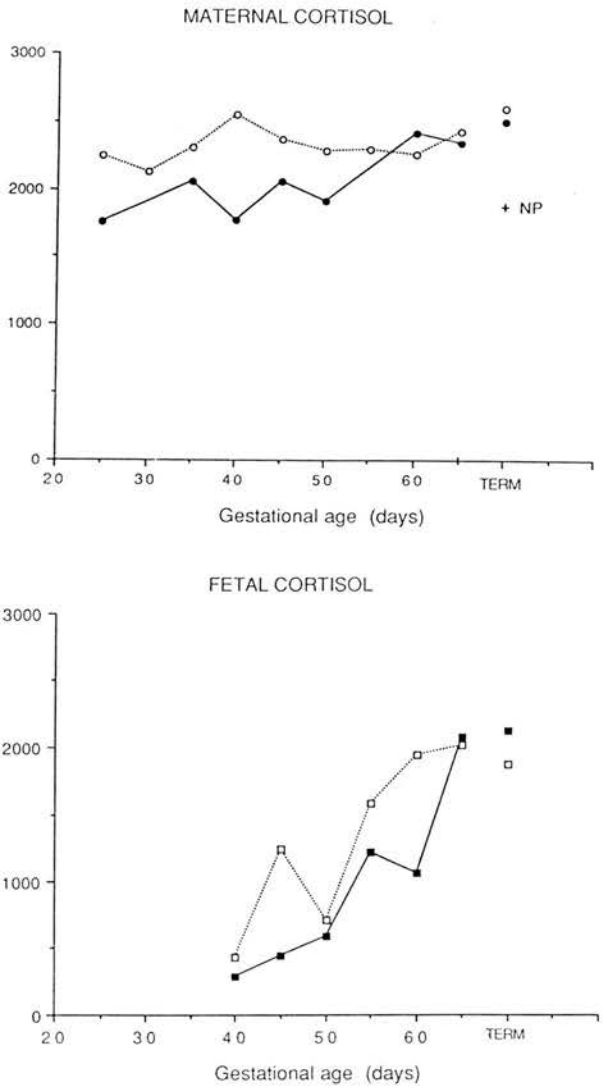
effects of maternal undernutrition on serum cor-
is shown in Fig. 7. Serum cortisol levels in
icted pregnant animals were higher than non-
nant levels at day 25 gestation and were
tained at this level throughout gestation. Control
s were similar to non-pregnant levels until day 50 of gestation when they began to rise, equalling levels seen in restricted dams by day 60. Calculation of regression coefficients revealed a significant difference between control and restricted dams (P<0.05). Fetal cortisol levels were very low when first measurable, on day 40 of gestation, and rose to reach levels seen in non-pregnant adults by birth. Serum cortisol concentrations of both restricted dams and fetuses tended to be higher than controls.

The correlation of cortisol levels with serum IGF levels was investigated. In maternal serum cortisol was negatively correlated with IGF-I levels in controls (r=–0.8114, P<0.05) and when all animals were considered (r=–0.6967, P<0.001). Cortisol was not correlated with levels of IGF-II. A similar pattern was seen in fetal serum where there was a negative correlation with IGF-I (r=–0.5478, P<0.05), but no correlation with IGF-II levels.

Discussion

Maternal and fetal weights

In *ad lib* fed animals net maternal body weight increases until approximately day 45, or the end of the second trimester. Maternal voluntary feed intake also



. Changes in cortisol concentration of (a) maternal, and (b) era with gestational age. Key as Fig. 3.

ases immediately after conception reaching a pla- at day 45 (personal observation). Conceptus ht, however, has a negligible impact until about 5 of gestation, accounting for less than 1% of total rnal body weight at day 25 (Fig. 1a). This early ase in weight and food consumption seems to be ciated with enhanced fat deposition in the mater- body in the first half of gestation (Flint, ett-Smith, Clegg & Vernon, 1979). This is mo- ed in the latter stages of gestation (Morgan & mith, 1977; Flint *et al.*, 1979; Close, Noblet & ens, 1984) to support fetal growth. In the re- ted dams there was no increase in net maternal weight in the first half of pregnancy (Fig. 1b) esting that there was no extra fat deposition in and mid pregnancy. This indicates that dams ed to mobilize their own pre-pregnancy fat stores the last half of gestation which resulted in the loss out 10% of their body weight. In the pregnant rat arge retroperitoneal and perirenal fat deposits in adequately nourished dams are absent in restricted animals (Berg, 1965), suggesting maternal fat mobilization occurs with nutritional restriction.

A 40% reduction in maternal intake in this study resulted in a similar degree of reduction in fetal growth as seen in other studies (Ward & Stickland, 1991; Dwyer & Stickland, 1992). Calculation of regression lines (Fig. 2) indicated that this occurred by a lower growth rate in the restricted fetuses throughout gestation. Muscle weights seemed to be disproportionately affected by undernutrition being reduced by approximately 45% by late gestation whereas body weight was reduced by only 38%.

Serum IGFs

In this study maternal levels of IGF-I were elevated with respect to non-pregnant animals at day 25, the end of the first trimester, and declined to non-pregnant levels by term (Fig. 3a). Control levels were higher in early gestation but groups were indistinguishable by the last trimester. A similar pattern of IGF-I variation with gestational age is seen in mouse and rat dams, with elevated IGF-I in the first half of gestation and a decline in late gestation (D'Ercole & Underwood, 1980; Sheppard & Bala, 1986; Gargosky, Walton, Owens, Wallace & Ballard, 1990; Davenport, Clemmons, Miles, Camacho-Hubner, D'Ercole & Underwood, 1990b; Donovan, Giudice, Murphy, Hintz & Rosenfeld, 1991). In the sheep, however, there is no change in IGF-I level with pregnancy (van Vliet, Styne, Kaplan & Grumbach, 1983), and in human and primate pregnancy IGF-I increases with gestation (Furlanetto, Underwood, Van Wyk & Handwerger, 1978; Bala, Lopatka, Leung, McCoy & McArthur, 1981; Wilson, Bennett, Adamson, Nagashima, Liu, DeNatale, Hintz & Rosenfeld, 1982; Hall, Enberg, Hellem, Lundin, Ottosson-Seeberger, Sara, Trygstad & Ofverholm, 1984; Styne, 1991). These differences may be related to the size and weight of the litter carried by each species: in humans progeny weigh 5–6% of the maternal body weight, in sheep 10–15%, in mice and rats approximately 20–25%, and in the guinea pig as much as 50% (figures from Leitch, Hytten & Billewicz, 1959). This suggests that in the rodents, especially the guinea pig, the litter is making a larger nutritional demand on the mother, and this may explain the decline in serum IGF-I. This is supported by the negative correlation between maternal serum IGF-I and increasing fetal, litter and placental weights seen in this study (Table 1).

This study has also demonstrated that control maternal levels of IGF-I were higher than restricted levels until the last trimester (Fig. 3a). IGF-I is known to play a role in glucose clearance and protein metabolism (Gluckman, Douglas, Ambler, Breier, Hodgkinson, Koea & Shaw, 1991) and, in starved mice, IGF-I administration reduces weight loss (O'Sullivan, Gluckman, Breier, Woodall, Siddiqui & McCutcheon, 1989). The reduced increase in serum IGF-I in mid-gestation in the restricted dams, therefore, may be

ociated with the lack of increase in net maternal
ly weight seen in these animals (Fig. 1b). This is
pported by the correlation between net maternal
ly weight and serum IGF-I. In addition to the role
um IGF-I may play in maternal metabolism, it may
o have an important endocrine role in affecting the
wth of the feto-placental unit. In mouse lines in-
d for high and low serum IGF-I concentrations, the
vated IGF-I in the high line dams produced heavier
al and placental weights than controls (Kroonsberg,
Cutcheon, Siddiqui, MacKenzie, Blair, Ormsby,
eier & Gluckman, 1989). It therefore appears likely
t the reduced IGF-I in the restricted animals at
d-gestation may have a detrimental effect on
cental and fetal growth.

Fetal IGF-I levels were considerably lower than both
ternal and non-pregnant adult levels and showed no
parent temporal regulation with gestational age
g. 3b). Low levels of fetal IGF-I have been seen in
ny species (Rat: Adams, Nissley, Handwerger & Re-
er, 1983; Guinea pig: Daughaday, Yanow & Kapida,
86; Sheep: Gluckman & Butler, 1983; Human: Gluck-
n *et al.*, 1983; Mouse: D'Ercole & Underwood, 1980;
: Spencer, Hallett, Beerman & MacDonald, 1989;
nkey: Liu, Powell, Styne & Hintz, 1991), and there-
e seem to be a common feature of fetal endocrinology.
ny studies have, however, demonstrated a positive
relation between serum IGF-I and fetal weight in
e gestation (Gluckman & Brinsmead, 1976; Jones *et*
1990; Hausman *et al.*, 1991), and a tendency to-
rds that relationship was seen in the day 65 fetuses in
s study.

erum IGF-I tended to be decreased in the restricted
ises throughout gestation (Fig. 3b). IUGR by
rine artery ligation, caruncleclectomy in sheep,
ternal fasting or by unknown means in human
gnancy all result in reduced fetal serum IGF-I (De
ns, Hill, Fekette, Robson, Fieller, van Assche &
ner, 1984; Vilesis & D'Ercole, 1986; Jones *et al.*,
7; 1988; 1990; Davenport *et al.*, 1990a; Straus, Ooi,
owski & Rechler, 1991). The results presented here
gest that chronic maternal undernutrition also
ses a depression of fetal serum IGF-I.

Maternal serum IGF-II concentration was similar to
-pregnant levels throughout gestation and showed
pparent change with gestational age or nutritional
tus (Fig. 4a). Other studies in the rat, guinea pig
human have shown that maternal IGF-II is low
similar to, or slightly higher than, non-pregnant
ls (Bala *et al.*, 1981; Daughaday, Parker, Borow-
, Trivedi & Kapadia, 1982; Daughaday *et al.*, 1986;
gosky *et al.*, 1990). In the pregnant rat a three day
causes reduced maternal serum IGF-I, but not
-II (Davenport *et al.*, 1990a), and in the non-preg-
t animal a similar fast also leaves serum IGF-II
ffected (Davenport, Svoboda, Koerber, Van Wyk,
mmons & Underwood, 1988). Maternal protein re-
ction, however, is reported to reduce maternal
-II in the pregnant rat (Pilistine *et al.*, 1984). It is
possible that the degree of undernutrition used in the present study was not severe enough, or of long enough duration, to cause a depression of maternal IGF-II.

Fetal serum IGF-II concentration was similar to maternal levels for much of gestation, except for the period between gestational days 50 and 65 when there was a large increase in IGF-II in the control group and a smaller peak in the restricted group (Fig. 4b). Peak levels of IGF-II around day 55 in the guinea pig have been seen by other groups (Daughaday *et al.*, 1986; Lafeber *et al.*, 1987) and seem to coincide with the time of maximum hepatic glycogen deposition (Jones, 1976). The depression of the peak seen in the restricted fetuses may be associated with reduced glycogen deposition in the livers of these animals. Maternal fasting for 5 days in the rat or 2 days in the mid-gestational guinea pig causes a reduction in fetal serum IGF-II (Jones *et al.*, 1990; Straus *et al.*, 1991), as does maternal starvation in the late gestation ewe (Gluckman & Butler, 1985) and prolonged protein deficiency in the rat (Pilistine *et al.*, 1984). IUGR by artery ligation or caruncleclectomy in sheep and guinea pig, however, have been found to cause an elevation of fetal IGF-II (Jones *et al.*, 1987; 1988; 1990) as does artery compression or adrenaline infusion (Jones *et al.*, 1988). IGF-II levels, therefore, seem to be elevated by conditions causing hypoxia in the fetus, and depressed by a reduced nutrient supply.

The precise role of IGF-II is not known, however there is some evidence that IGF-II may be closely involved in glucose metabolism. Glucose infusion into sheep or guinea pigs causes an increase in fetal IGF-II (Gluckman & Butler, 1985; Jones *et al.*, 1990), and fetal IGF-II is correlated with hepatic glycogen concentration (Jones *et al.*, 1990). In the day 65 guinea pigs in this study serum IGF-II tended to be related to fetal, placental and biceps weights. In addition, IGF-II deficient mouse mutants were 40% smaller than the wild type at birth (Dechiara *et al.*, 1990), indicating a significant influence of IGF-II on fetal growth. It is possible that IGF-II plays a permissive role in fetal growth by mediating glucose availability; this would explain the apparently greater influence of IGF-II in the fetus which has a greater requirement for glucose (Jones, 1976) than the adult.

Thyroid hormones

Maternal T3 was lower than non-pregnant levels and declined slightly with gestational age (Fig. 5a). Maternal T4 also declined with gestational age, from non-pregnant levels at day 25 (Fig. 6a). Control levels tended to be higher than restricted levels. In the pig and human a similar decline in maternal serum T4 is also seen with increasing gestational age (Atinmo, Baldijao, Pond & Barnes, 1976; Geissler, Margen & Calloway, 1979). These decreases probably reflect the increasing nutritional burden placed on the mother by the developing fetuses, since a major role of the thyroid hormones is to regulate metabolic rate and preserve

erall body tissues (reviewed by Danforth & Burger, 89). This is supported by a significant negative rrelation between litter weight and serum T3 or T4. tal serum T3 and T4 were very low in mid gestation t began to rise during the last trimester reaching gh levels at term (Fig. 5b & 6b). Control levels of T4 ded to be higher than restricted, especially at term. e passage of T4 over the placenta is known to rease with gestational age in the sheep, guinea pig, bit and man (Osorio & Myant, 1960; London, ney & Rawson, 1963; Comline, Nathanielsz & Sil-, 1970), however T3 is not thought to be transferred ndon *et al.*, 1963). Maternal transport may account some of the increase in T4 in late gestation fetal um seen here and in many species (Pig: Atinmo *et* 1976; Human, sheep, cow, horse: Parker, Williams, erne & Young, 1980; Rat: Wrutniak & Cabello, 83; Nathanielsz, 1975). This may also reflect the e-dependent increase in thyroid hormone depend-cy for growth seen in many species (Osorio & Myant, 60). The thyroid hormones seem to be important late fetal growth (Cooke, Yonemura & Nicoll, 1984).

The thyroid hormones have also been implicated in regulation of IGFs. They are reported to affect the I- stimulation of IGF-I (Wolf, Ingbar & Moses, 1989) to act directly in tissue culture systems (Binoux, vre-Bauman, Lassarre, Barret & Tixier-Vidal, 85; Ikeda *et al.*, 1989). Several studies have shown t T4 alone, or with GH, can stimulate IGF-I to rmal levels in hypophysectomised adult rats (Gas-d *et al.*, 1978; Fagin *et al.*, 1989). However, cultured man fetal fibroblasts did not produce IGF-I in ponse to thyroxine treatment (Clemmons, Under-od & Van Wyk, 1981). Results presented here also monstrate a difference between fetal and maternal a, since T4 and T3 were correlated with IGF-I in the lt, but there was no correlation in the fetus. This y be related to the late dependency of fetal growth the thyroid hormones (Cooke *et al.*, 1984).

um cortisol levels

um cortisol from control guinea pig dams was simi- to non-pregnant concentrations until the last nester when levels increased (Fig. 7a). Serum cor-l was elevated in restricted dams on day 25 of tation and was maintained at that level throughout tation. In the human and guinea pig maternal tisol is known to rise towards the end of gestation issler *et al.*, 1979; Rosenthal, Slaunwhite & Sand-g, 1969; Jones, 1974). Fasting or malnutrition also ses a rise in plasma cortisol in humans and sheep ith, Bledsoe & Chhetri, 1975; Bassett & Madill, 4). These results are consistent with the known cts of cortisol in simulating gluconeogensis, mobi-ng fat from adipose tissue and promoting ogenesis. If the serum cortisol levels seen in this dy reflect the degree of mobilization of fat reserves, appears that considerable mobilization of fat erves was already occurring at day 25 in restricted dams, and these levels were maintained through pregnancy. In the control animals metabolic changes to support fetal growth only occurred in late gestation.

Fetal cortisol was low at day 40, particularly in the control group, and increased to term (Fig. 7b). Levels from restricted fetuses were always higher than controls. A prepartum rise in cortisol is classically seen in many species (Sheep: Bassett & Thorburn, 1969; Robinson, Hart, Kingston & Jones, 1980; Rabbit: Mulay, Giannopoulos & Solomon, 1973; Guinea pig: Jones 1974; Rat: Ota, Ota & Yokoyama, 1974; Pig: Spencer *et al.*, 1989), and is implicated in the initiation of labour and parturition (Bassett & Thorburn, 1969). The elevated levels of cortisol in the restricted fetuses may, therefore, explain the early littering which sometimes accompanies maternal food restriction (personal observation). Glucocorticoids are also thought to be involved in glycogen deposition in the guinea pig liver (Jones, 1974) and induction of lung surfactant in the rabbit (Mulay *et al.*, 1973). In addition, a low birth weight in the pig is associated with high serum cortisol (Wise, Stone & Vernon, 1991). This may be related to the influence of the glucocorticoids on IGF levels, since glucocorticoid treatment causes a reduction in IGF-II mRNA in the neonatal rat liver (Beck *et al.*, 1988; Levinovitz & Norstedt, 1989). In this study maternal and fetal serum IGF-I were inversely correlated with serum cortisol, suggesting that there may be a relationship between levels of cortisol and the IGFs.

In conclusion, in this study a reduction in maternal feed intake of 40% throughout gestation had a similar effect on fetal and muscle development to that seen elsewhere (Ward & Stickland, 1991; Dwyer & Stickland, 1992). The effect of undernutrition on maternal serum IGF-I suggests that this may have an important effect on placental growth, and indirectly on fetal growth. Maternal IGF-II seems less likely to mediate the effects of undernutrition on fetal development. Fetal serum IGF-I, and to some extent IGF-II, may also mediate maternal undernutrition. This may indicate a mechanism by which myoblast proliferation is reduced in these animals since the IGFs stimulate myoblast proliferation *in vitro* (Ewton & Florini, 1980). Fetal IGF-II seems to be mainly related to hepatic glycogen deposition, but may also have a role to play in the increase in muscle cell number. Thyroid hormones do not appear to be important for muscle growth of the fetal guinea pig until late gestation or early in postnatal life. This is consistent with the negative proliferative effects obtained by adding thyroid hormones to myoblast cells in culture (Florini, 1987). Levels of the glucocorticoids, however, were correlated with serum IGF levels and may serve to regulate expression of IGF mRNA. However, serum levels are mainly a reflection of hepatic IGF production (D'Ercole, Stiles & Underwood, 1984) and, hence, the endocrine action of the IGFs. Since the IGFs are also thought to have a major paracrine activity this may not be an accurate representation of the effects of

utrition on IGF action at the muscle itself. Measurement of the tissue levels of the IGFs in undernourished developing muscle may help to give a fuller picture of the effects of maternal undernutrition on fetal muscle development. In addition, nutrition and glucocorticoid levels are known to affect maternal and fetal levels of the IGF binding proteins (Gallaher, Breier, Oliver, Harding & Gluckman, 1992; Price, Stiles, Moatsaats & D'Ercole, 1992). This will have an influence on the circulating levels of the IGFs, and may act as a regulatory mechanism.

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eferences

lams, S.O., Nissley, S.P., Handwerger, S., & Rechler, M.M. (1983) Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts. *Nature*, **302**, 150-152.

hton, I.K., Zapf, J., Einschenk, I., & MacKenzie, I.Z. (1985) Insulin-like growth factors (IGF) 1 & 2 in human fetal plasma and relationship to gestational age and fetal size during mid-pregnancy. *Acta Endocrinologica*, **110**, 558-563.

inmo, T., Baldijao, C., Pond, W.G. & Barnes, R.H. (1976) The effect of dietary protein restriction on serum thyroxine levels of pregnant and growing swine. *Journal of Nutrition*, **108**, 1456-1533.

la, R.M., Lopatka, J., Leung, A., McCoy, E. & McArthur, R.G. (1981) Serum immunoreactive somatomedin levels in normal adults, pregnant women at term, children at various ages and children with constitutionally delayed growth. *Journal of Clinical Endocrinology and Metabolism*, **52**, 508-512.

ssett, J.M. & Madill, D. (1974) The influence of maternal nutrition on plasma hormone and metabolite concentrations of fetal lambs. *Journal of Endocrinology*, **61**, 465-477.

ssett, J.M. & Thorburn, G.D. (1969) Fetal plasma corticosteroids and initiation of parturition in sheep. *Journal of Endocrinology*, **44**, 285-286.

ck, F., Samani, N.J., Senior, P., Byrne, S., Morgan, K., Gebhard, R. & Brammar, W.J. (1988) Control of IGF-II mRNA levels by glucocorticoids in the neonatal rat. *Journal of Molecular Endocrinology*, **1**, R5-R8.

nnett, A., Wilson, D.M., Liu, F., Nagashima, R., Rosenfeld, R.G. & Hintz, R.L. (1983) Levels of the insulin-like growth factors I and II in human cord blood. *Journal of Clinical Endocrinology and Metabolism*, **57**, 609-612.

rg, B.N. (1965) Dietary restriction and reproduction in the rat. *Journal of Nutrition*, **87**, 344-348.

noux, M., Faivre-Bauman, A., Lassarre, C., Barret, A. & Tixier-Vidal, A. (1985) Triiodothyronine stimulates the production of IGF by fetal hypothalamus cells cultured in serum-free medium. *Developmental Brain Research*, **21**, 319-321.

wsher, R.R., Lee, W-H., Apathy, J.M., O'Brien, P.J., Ferguson, A.L. & Henry, D.P. (1991) Measurement of IGF-II in physiological fluids and tissues. I. An improved extraction procedure and radioimmunoassay for human and rat fluids. *Endocrinology*, **128**, 805-814.

ionomo, F.C., Grohs, D.L., Baile, C.A. & Campion, D.R. (1988) Determination of circulating levels of IGF-II in swine. *Domestic Animal Endocrinology*, **5**, 323-329.

emmons, D.R., Underwood, L.E. & Van Wyk, J.J. (1981) Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. *Journal of Clinical Investigation*, **67**, 10-19.

ose, W.H., Noblet, J. & Heavens, R.P. (1984) The partition of body weight gain in the pregnant sow. *Livestock Production Science*, **11**, 517-527.

mline, R.S., Nathanielsz, P.W. & Silver, M. (1970) Passage of thyroxine across the placenta in the fetal sheep. *Journal of Physiology*, **207**, 3-4P.

Cooke, P.S., Yonemura, G.U. & Nicoll, C.S. 1984) Development of thyroid hormone dependence for growth in the rat: a study involving transplanted fetal, neonatal and juvenile tissues. *Endocrinology*, **115**, 2059-2064.

D'Ercole, A.J., Stiles, A.D. & Underwood, L.E. (1984) Tissue concentrations of somatomedin-C: Further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *Proceedings of the National Academy of Sciences*, **81**, 935-939.

D'Ercole, A.J. & Underwood, L.E. (1980) Ontogeny of somatomedin during development in the mouse. *Developmental Biology*, **79**, 33-45.

Danforth, E. & Burger, A.G. (1989) The impact of nutrition on thyroid hormone physiology and action. *Annual Review of Nutrition*, **9**, 201-227.

Daughaday, W.H., Kapadia, M. & Mariz, I. (1987) Serum somatomedin binding proteins: physiological significance and interference in radioligand assay. *Journal of Laboratory and Clinical Medicine*, **109**, 355-363.

Daughaday, W.H., Parker, K.A., Borowsky, S., Trivedi, B. & Kapadia, M. (1982) Measurement of somatomedin-related peptides in fetal, neonatal and maternal rat serum by IGF-I RIA, IGF-II RRA & MSA RRA after acid-ethanol extraction. *Endocrinology*, **110**, 575-581.

Daughaday, W.H., Yanow, C.E., & Kapida, M. (1986) Insulin-like growth factors I and II in maternal and fetal guinea-pig serum. *Endocrinology*, **119**, 490-494.

Davenport, M.L., Clemmons, D.R., Miles, M.V., Comacho- Hubner, C., D'Ercole, A.J. & Underwood, L.E. (1990b) Regulation of serum IGF-I and IGF-binding proteins during rat pregnancy. *Endocrinology*, **127**, 1278-1286.

Davenport, M.L., D'Ercole, A.J. & Underwood, L.E. (1990a) Effects of maternal fasting on fetal growth, serum IGFs and tissue IGF mRNAs. *Endocrinology*, **126**, 2062-2067.

Davenport, M.L., Svoboda, M.E., Koerber, K.L., Van Wyk, J.J., Clemmons, D.R. & Underwood, L.E. (1988) Serum concentrations of IGF-II are not changed by short term fasting and refeeding. *Journal of Clinical Endocrinology and Metabolism*, **67**, 1231-1236.

Dechiara, T.M., Efstratiadis, A., & Robertson, E.J. (1990) A growth-deficiency phenotype in heterozygous mice carrying an IGF-II gene disrupted by targeting. *Nature*, **345**, 78-80.

Deprins, F.A., Hill, D.J., Fekete, M., Robsen, D.J., Fieller, N.R.J., van Assche, F.A. & Milner, R.D.G. (1984) Reduced plasma somatomedin activity and costal cartilage sulfate incorporation activity during experimental growth retardation in the fetal rat. *Pediatric Research*, **18**, 1100-1104.

Donovan, S.M., Giudice, L.C., Murphy, L.J., Hintz, R.L. & Rosenfeld, R.G. (1991) Maternal IGFBP mRNA during rat pregnancy. *Endocrinology*, **129**, 3359-3366.

Dwyer, C.M. & Stickland, N.C. (1992) Does the anatomical location of a muscle affect the influence of undernutrition on muscle fibre number? *Journal of Anatomy*, **181**, 373-376.

Eigenmann, J.E., Zanesco, S., Arnold, U., & Froesch, E.R. (1984) Growth hormone and insulin-like growth factor I in German Shepherd dwarf dogs. *Acta Endocrinologica*, **105**, 289-293.

Evans, H.E. & Sack, W.O. (1973) Prenatal development of domestic and laboratory animals: growth curves, external features and selected references. *Zentralblatt für Veterinaermedizin*, **2**, 11-45.

Ewton, D.Z. & Florini, J.R. (1980) Relative effects of the somatomedins, MSA, and growth hormone on myoblasts and myotubes iin culture. *Endocrinology*, **106**, 577-583.

Fagin, J.A., Fernandez-Mejia, & Melmed, S. (1989) Pituitary IGF-I gene expression: regulation by triiodothyronine and growth hormone. *Endocrinology*, 125, 2385-2391.

Falconer, J., Forbes, J.M., Hart, I.C., Robinson, J.S., & Thorburn, G.D. (1979) Somatomedin-like activity (SLA) in plasma after fetal hypophysectomy or nephrectomy and in experimental intrauterine growth retardation in sheep. *Journal of Endocrinology*, **83**, 119-127.

Fernandez, S.F., Menendez, M.F, Fernandez, B.M. & Patterson, A.M. (1985) Malnutrition *in utero* and during lactation in the rat: Relationship of dams' weight gain and development of suckling. *Nutrition Research*, **5**, 413-421.

Flint, D.J., Sinnett-Smith, P.A., Clegg, R.A. & Vernon, R.G. (1979) Role of insulin receptors in the changing metabolism of adipose tissue during pregnancy and lactation in the rat. *Biochemistry Journal*, **182**, 421-427.

ini, J.R. (1987) Hormonal control of muscle growth. *Muscle and erve*, **10**, 577-598.
anetto, R.W., Underwood, L.E., Van Wyk, J.J. & Handwerger, (1978) Serum somatomedin-C is elevated late in pregnancy. *urnal of Clinical Endocrinology and Metabolism*, **47**, 695-698.
aher, B.W., Breier, B.H., Oliver, M.H., Harding, J.E. & Gluck-an, P.D. (1992) Ontogenic differences in the nutritional regula-on of circulating IGF binding proteins in sheep plasma. *Acta ndocrinologica*, **126**, 49-54.
gosky, S.E., Walton, P.E., Owens, P.C., Wallace, J.C. & Ballard, J. (1990) IGF-I and IGF-binding proteins in the rat during late egnancy. *Journal of Endocrinology*, **127**, 383-390.
oard, T., Wondergem, R., Hamamdzic, M. & Klitgaard (1978) erum somatomedin stimulation in thyroxine-treated hypo-ysectomised rats. *Endocrinology*, **102**, 606-611.
sler, C., Margen, S. & Calloway, D.H. (1979) Lactation and egnancy in Iran III. Hormonal factors. *American Journal of inical Nutrition*, **32**, 1097-1111.
ckman, P.D. & Brinsmead, M.W. (1976) Somatomedin in cord ood: relationship to gestational age and birth size. *Journal of inical Endocrinology and Metabolism*, **43**, 1378-1381.
ckman, P.D. & Butler, J.H. (1983) Parturition-related changes insulin-like growth factors-I and -II in the perinatal lamb. *urnal of Endocrinology*, **99**, 223-232.
ckman, P.D. & Butler, J.H. (1985) Insulin-like growth factors in e fetus. In *The Physiological Development of the Fetus and the ewborn* (Jones, C.T. & Nathanielsz, P.W., eds.). Academic Press, ndon.
ckman, P.D., Douglas, R.G., Ambler, G.R., Breier, B.H., Hodg-nson, S.C., Koea, J.B. & Shaw, J.H.F. (1991) The endocrine role IGF-I. *Acta Paediatrica Scandinavia*, [Suppl] **372**, 97-105.
ckman, P.D., Johnson-Barrett, J.J., Butler, J.H., Edgar, B.W. & nn, T.R. (1983) Studies of IGF-I and -II by specific radioligand says in umbilical cord blood. *Clinical Endocrinology*, **19**, 405-3.
, K., Enberg, G., Hellem, E., Lundin, G., Ottoson- Seeberger, A., ra, V., Trtgstad, O. & Ofverholm, U (1984) Somatomedin levels pregnancy: Longitudinal study in healthy subjects and patients th growth hormone deficiency. *Journal of Clinical Endocrino-gy and Metabolism*, **59**, 587
sman, G.J., Campion, D.R. & Buonomo, F.C. (1991) Concentra-n of IGF-I and IGF-II in tissues of developing lean and obese g foetuses. *Growth, Development and Aging*, **55**, 43-52.
a, T., Fujiyama, K., Tekeuchi, T., Honda, M., Mokuda, O., minga, M. & Mashiba, H. (1989) Effect of thyroid hormone on matomedin-C release from perfused rat liver. *Experientia*, **45**, 0-171.
s, C.T. (1974) Corticosteroid concentrations in the plasma of etal and maternal guinea pigs during gestation. *Endocrinology*, , 1129-1133.
s, C.T. (1976) Fetal metabolism and fetal growth. *Journal of production and Fertility*, **47**, 189-201.
s, C.T., Gu, W., Harding, J.E, Price, D.A. & Parer, J.T. (1988) udies on the growth of the fetal sheep. Effects of surgical duction in placental size, or experimental manipulation of erine blood flow on plasma sulphation promoting activity and the concentration of IGF-I and -II. *Journal of Developmental ysiology*, **10**, 179-189.
s, C.T., Lafeber, H.N., Price, D.A., & Parer, J.T. (1987) Studies the growth of the fetal guinea pig. Effects of the reduction in e uterine blood flow on the plasma sulphation-promoting activ-and on the concentration of the insulin-like growth factors -I d -II. *Journal of Developmental Physiology*, **9**, 181-201.
s, C.T., Lafeber, H.N., Rolph, T.P. & Parer, J.T. (1990) Studies the growth of the fetal guinea pig. The effects of nutritional nipulation on prenatal growth and plasma somatomedin activ-and IGF concentrations. *Journal of Developmental Physiology*, , 189-197.
s, C.T., Rolph, T.P., Lafeber, H.N., Gu, W., Harding, J.E. & rer, J.T. (1985) Experimental studies on the control of fetal owth. In *The Physiological Development of the Fetus and New-rn* (Jones, C.T & Nathanielsz, P.W., eds.) Academic Press, ndon.
nsberg, C., McCutcheon, S.N., Siddiqui, R.A., MacKenzie, O.S., Blair, H.T., Ormsby, J.E., Breier, B.H. & Gluckman, P.D. 89) Reproductive performance and fetal growth in female mice m lines divergently selected on the basis of plasma IGF-I ncentrations. *Journal of Reproduction and Fertility*, **87**, 349-353
Lafeber, H.N., Jones, C.T. & Price, D.A. (1987) Studies on the growth of the fetal guinea pig. Changes in the plasma concentration of sulphation-promoting activity and of IGFs during gestation. *Journal of Developmental Physiology*, **9**, 169-179.
Lafeber, H.N., Rolph, T.P. & Jones, C.T. (1984) Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *Journal of Developmental Physiology*, **6**, 441-459.
Leitch, I., Hytten, F.E. & Billewicz, W.Z. (1959) The maternal and neonatal weights of some mammalia. *Proceedings of the Zoological Society of London*, **133**, 11-28.
Levinovitz, A. & Norstedt, G. (1989) Developmental and steroid hormonal regulation of IGF-II expression. *Molecular Endocrinology*, **3**, 797-804.
Liu, F., Powell, D.R., Styne, D.M. & Hintz, R.L. (1991) Insulin-like growth factors and IGF-binding proteins in the developing Rhesus monkey. *Journal of Clinical Endocrinology and Metabolism*, **72**, 905-911
London, W.T., Money, W.L. & Rawson, R.W. (1963) Placental transport of I131-labelled thyroxine and triiodothyronine in the guinea pig. *Endocrinology*, **73**, 205-209.
Merrimee, T.J., Zapf, J. & Froesch, E.R. (1982) IGFs in pregnancy: studies in a growth hormone deficient dwarf. *Journal of Clinical Endocrinology and Metabolism*, **54**, 1101-1103.
Morgan, B.L.G. & Naismith, D.J. (1977) Effects on the products of conception of protein supplementation of the diets of rats. *Journal of Nutrition*, **107**, 1590-1594.
Morrell, D.J., Dadi, H., More, J., Taylor, A.M., Dabestani, A., Buchanan, C.R., Holder, A.T. & Preece, M.A. (1989) A monoclonal antibody to human IGF-I: characterization, use in radioimmunoassay and effect on the biological activities of the growth factor. *Journal of Molecular Endocrinology*, **2**, 201-206.
Mulay, S., Giannopoulos, G. & Solomon, S. (1973) Corticosteroid levels in the mother and fetus of the rabbit during gestation. *Endocrinology*, **93**, 1342-1348.
Nathanielsz, P.W. (1975) Thyroid function in the fetus and newborn mammal. *British Medical Bulletin*, **31**, 51-57.
O'Sullivan, U., Gluckman, P.D., Breier, B.H., Woodall, S., Siddiqui, R.A. & McCutcheon, S.N. (1989) IGF-I in mice reduces weight loss during starvation. *Endocrinology*, **125**, 2793-2794.
Ogata, E.S., Bussey, M.E., Labarbera, A. & Finley, S. (1985) Altered growth, hypoglycaemia, hypoalaninemia & ketonemia in the young rat: Postnatal consequences of IUGR. *Pediatric Research*, 19, 32-37.
Osorio, C. & Myant, N.B. (1960) The passage of thyroid hormone from mother to foetus and its relationship to foetal development. *British Medical Bulletin*, **16**, 159-164.
Ota, K., Ota, T. & Yokoyama, A. (1974) Plasma corticosterone concentrations and pituitary prolactin content in late pregnancy and their within-day fluctuations in the rat. *Journal of Endocrinology*, **62**, 21-28.
Parker, R.O., Williams, P.E.V., Aherne, F.X. & Young, B.A. (1980) Postnatal changes in concentrations of serum and urinary thyroxine and 3,3',5-triiodothyronine in the pig. *Journal of Animal Science*, **51**, 132-137.
Pilistine, S.J., Moses, A.C. & Munro, H.N. (1984) Placental lactogen administration reverses the effect of low-protein diet on maternal and fetal serum somatomedin levels in the pregnant rat. *Proceedings of the National Academy of Sciences*, **81**, 5853-5857.
Price, W.A., Stiles, A.D., Moats-Staats, B.M. & D'Ercole, A. J. (1992) Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology*, **130**, 1424-1432.
Robinson, J.S., Hart, I.C., Kingston, E.J., Jones, C.T. & Thorburn, G.D. (1980) Studies on the growth of the foetal sheep. The effects of reduction of placental size on hormone concentration in fetal plasma. *Journal of Developmental Physiology*, **2**, 239-248.
Rosenthal, H.E., Slaunwhite, W.R. & Sandberg, A.S. (1969) Transcortin: A corticosteroid-binding protein of plasma. X Cortisol and progesterone interplay and unbound levels of these steroids in pregnancy. *Journal of Clinical Endocrinology*, **29**, 352-367.
Sheppard, M.S. & Bala, R.M. (1986) Profile of serum immunoreactive IGF-I during gestation in Wistar rats. *Canadian Journal of Physiology and Pharmacology*, **64,** 521-524.
Smith, S.R., Bledsoe, T. & Chhetri, M.K. (1975) Cortisol metabolism and the pituitary-adrenal axis in adults with protein-calorie malnutrition. *Journal of Clinical Endocrinology and Metabolism*, **40**, 43-52.

ncer, G.S.G., Hallett, K.G, Beerman, U. & MacDonald, A.A. 1989) Changes in the levels of growth hormones, insulin, cortisol, hyroxine and IGF-I with increasing gestational age in the fetal ig, and the effects of thyroidectomy *in utero*. *Comparative Biochemistry and Physiology*, **93A**, 467-472.

aus, D.S., Ooi, G.T., Orlowski, C.C. & Rechler, M.M. (1991) xpression of the genes for IGF-I, IGF-II and the IGFBPs 1 and in the fetal rat under conditions of IUGR caused by maternal sting. *Endocrinology*, **128**, 518-525.

ne, D.M. (1991) Serum IGF-I concentrations in the developing hesus monkey. *Journal of Medical Primatology*, **20**, 334-337.

Vliet, G., Styne, D.M., Kaplan, S.L. & Grumbach, M.M. (1983) ormone ontogeny in the ovine fetus. XVI. Plasma immunoreactve somatomedin-C/insulin-like growth factor I in the fetal and eonatal lamb and in the pregnant ewe. *Endocrinology*, **113**, 716-1720.

sis, R.A. & D'Ercole, A.J. (1986) Tissue and serum concentrations of Sm-C/IGF-I in fetal rats made growth retarded by uterine tery ligation. *Pediatric Research*, **20**, 126-130.

Ward, S.S. & Stickland, N.C. (1991) Why are fast and slow muscles differentially affected during prenatal undernutrition? *Muscle and Nerve*, **14**, 259-267.

Widdowson, E.M. (1971) Intrauterine growth retardation in the pig. *Biology of the Neonate*, **19**, 329-340.

Wilson, D.M., Bennett, A., Adamson, G.D., Nagashima, R.J., Hu, F., DeNatale, M.L., Hintz, R.L. & Rosenfeld, R.G. (1982) Somatomedins in pregnancy: A cross-sectional study of IGF-I and -II and somatomedin peptide content in normal human pregnancies. *Journal of Clinical Endocrinology and Metabolism*, **55**, 858-861.

Wise, T., Stone, R.T. & Vernon, M.W. (1991) Relationships of serum estriol, cortisol and albumin concentrations with pig weight at 110 days of gestation and at birth. *Biology of the Neonate*, **59**, 114-119.

Wolf, M., Ingbar, S.H. & Moses, A.C. (1989) Thyroid hormones and growth hormone interact to regulate IGF-I mRNA and circulating levels in the rat. *Endocrinology*, **125**, 2905-2914.

Wrutniak, C. & Cabello, G. (1983) Changes in the concentration of thyroxine in the plasma of rat fetuses during late gestation: influence of ligation of maternal uterine vein and artery. *Journal of Endocrinology*, **99**, 233-238.

Supplementation of a restricted maternal diet with protein or carbohydrate alone prevents a reduction in fetal muscle fibre number in the guinea-pig

BY CATHERINE M. DWYER AND NEIL C. STICKLAND

Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU

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A 60% reduction in maternal feed intake is known to cause a reduction of approximately 20% in *biceps brachii* fibre number in the guinea-pig fetus. This investigation was designed to isolate the dietary component responsible by reducing all dietary components to 60% of the *ad lib.* level and supplementing the protein, carbohydrate or fat component to the level of the *ad lib.* intake. Fetal muscles were examined at 50 d gestation to determine numbers of primary and secondary fibres, and at term to determine total fibre number. Fetal and neonatal weights were reduced in all restricted groups ($P < 0{\cdot}05$) when compared with *ad lib.* controls. At term this reduction was significantly less ($P < 0{\cdot}05$) in the protein-supplemented group (20%) than in the 60%-restricted and fat-supplemented groups (43%) and the carbohydrate-supplemented group (34%). *Biceps brachii* fibre numbers were reduced in the 60%-restricted and fat-supplemented groups by 14–16%, but fibre numbers were similar in control, protein-supplemented and carbohydrate-supplemented groups. Any reduction in fibre number was in the secondary fibre component of total fibre number. Therefore, *biceps brachii* fibre numbers were reduced only when maternal diets were deficient in both protein and carbohydrate.

Protein: Carbohydrate: Malnutrition: Fetal myofibre development: Guinea-pig

A 60% reduction in maternal feed intake is known to cause a reduction of approximately 20% in muscle fibre number in fast muscles (Ward & Stickland, 1991; Dwyer & Stickland, 1992). This reduction is in the secondary fibre component of total fibre number (Ward & Stickland, 1991). Secondary myotubes form as the second generation of muscle fibres, using the primary fibres as a framework on which to develop, and are considered more labile to nutritional influence than primaries (Handel & Stickland, 1987). The purpose of the present study was to determine which component of the maternal diet is most important in affecting the development of muscle fibre numbers.

Severe restriction of the protein component of the maternal diet (to 5 g/kg) in pigs results in a reduction of fetal weights, piglet birth weights, weaning weights and postnatal weight gains (Pond *et al.* 1968, 1987, 1991; Atinmo *et al.* 1974) and this stunting seems to be permanent (Pond & Wu, 1981; Pond *et al.* 1990). In the rat severe protein restriction caused smaller carcase and organ weights in late gestation fetuses (Zeman & Stanbrough, 1969). Mild protein restriction, however, has no effect (Pond, 1973). Energy restriction alone, with normal levels of protein, causes a greater reduction in fetal rat body weight than protein restriction alone (Anugwa & Pond, 1989), although this is not consistently the case in the pig (Pond *et al.* 1987). When non-protein energy restriction causes a reduction in piglet birth weight, this is associated with a low postnatal growth rate (Pond *et al.* 1987).

These findings, however, do not provide much information about the effect of these diets on the muscle cellularity of the developing fetuses. The effects of protein or carbohydrate

restriction in postnatal rats and children suggest that a low-protein diet leads to a reduced protein synthetic capability (Young & Alexis, 1968), therefore affecting cellular hypertrophy. A low-carbohydrate diet, however, causes reduced DNA levels but almost normal protein:DNA values (Cheek & Hill, 1970; Winick, 1970), therefore affecting hyperplasia of cells.

The present hypothesis was that the carbohydrate portion of the diet would affect the hyperplastic phase and, hence, myofibre number; the protein portion would affect only the hypertrophic phase, and not myofibre number. In the present experiment the diets were designed to supplement the individual component under study to *ad lib.* levels whilst all other nutritional components were reduced to 60% of an *ad lib.* intake. It should be emphasized, therefore, that supplemented dietary components were high only in relation to the other restricted components and did not exceed the levels eaten by the control animals.

MATERIALS AND METHODS

This project involved thirty-one pregnant Dunkin-Hartley guinea-pigs obtained from Bantam and Kingman Ltd (Aldbrough, Humb.). Guinea-pigs were in their second parity and weighed between 700 and 900 g at the start of the experiment. On day 2 of gestation females were housed individually in plastic cages and assigned to a nutritional treatment. Diets were based on SG1 pellets (Grain Harvesters, Wingham, Kent) which provide (g/kg): protein 190, starch and sugars 232·5, oil 42·5, fibre 112·5, digestible energy 9·60 MJ. Three test diets were formulated which were designed to provide control levels of the component under study and 60% of *ad lib.* (negative control) levels of all other components (Table 1). The compositions of these diets were as follows (g/kg): R+P, protein 273, starch and sugars 232·5, oil 42·5; R+C, protein 190, starch and sugars 335, oil 42·5; R+F, protein 190, starch and sugars 232·5, oil 91.

All diets were enriched with vitamin C. Pregnant guinea-pigs were randomly assigned to treatments as follows: (1) Controls, *ad lib.* access to SG1 pellets, *n* 6; (2) 40R, pair-fed with SG1 pellets at 60% of *ad lib.* intake, *n* 6; (3) R+P, protein supplemented, *n* 7; (4) R+C, carbohydrate supplemented, *n* 6; (5) R+F, fat supplemented, *n* 6.

All animals were given unlimited access to clean, fresh drinking water containing vitamin E (30 mg/l; Roche Products Ltd, Welwyn Garden City, Herts.) since they were not allowed access to hay for the duration of the experiment.

Three randomly selected pregnant animals from each nutritional group were killed at 50 d gestation by an intraperitoneal injection of sodium pentabarbitone (1 ml/kg body weight; Euthesate; Willows Francis Veterinary, a division of A. H. Robins, Crawley, West Sussex), and the remainder were allowed to litter and neonates were killed at term. Stillborn animals or pups which died within 24 h of birth were counted and weighed. Muscles were not always available from these animals, therefore, for consistency, muscles were analysed only from those animals which were alive at the time of sampling. *Biceps brachii* muscles were removed from all fetuses and neonates (*n* 99), mounted on cork chucks and rapidly frozen in hexane cooled in liquid N_2. Whole muscle transverse sections (10 μm) were cut at $-25°$ in a cryostat and stained for myosin ATPase (*EC* 3.6.1.32) activity after the method of Guth & Samaha (1970). At 50 d gestation all biceps muscle fibres are present but it is still possible to determine which fibres developed as primary and secondary fibres on the basis of their ATPase staining pattern (Ward & Stickland, 1991). Muscles from fetuses (*n* 57) were used to determine values for secondary:primary (S:P) fibres, and total fibre numbers. Neonates provided information about growth rate throughout gestation and muscles from neonates (*n* 42) were used to determine total myofibre numbers only, since cell lineages could no longer be distinguished at this age. All fibres were counted blind to

Table 1. *Example of the composition of diets eaten by different dietary groups, matched to a control animal eating 50 g feed* ad lib.*

Dietary group	Total feed (g/d)	Protein (g)	Carbohydrate (g)	Fat (g)	Total ME (KJ)
Control (*n* 6)	50	9·5	11·63	2·13	477·5
40R (*n* 6)	30	5·7	6·98	1·28	286·5
R+P (*n* 7)	33·8	9·23	6·98	1·28	363·2
R+C (*n* 6)	34·7	5·7	11·52	1·28	358·8
R+F (*n* 6)	30·9	5·7	6·98	2·90	345·4

ME, metabolizable energy; Control, *ad. lib.* access to SG1 pellets (Grain Harvesters, Wingham, Kent).
40R, pair-fed with SG1 pellets at 60% of *ad lib.* intake; R+P, protein-supplemented; R+C, carbohydrate-supplemented; R+F, fat-supplemented.
* For details of diets, see p. 174.

diet groups using a Seescan Image Analysis system (Seescan Plc, Cambridge). Nutritional groups were compared using Newman-Keul ANOVA statistics.

RESULTS

Maternal and fetal weight

The effect of nutritional treatments on maternal and fetal weight gain is shown in Table 2. At both 50 d gestation and term, control dams tended to gain more weight than any restricted group. Variations between individuals were, however, quite large. By term only the 40R group and the R+C groups showed a mean net weight loss when the influence of conceptus weight was removed (−23 and −3 g respectively). This was significantly different from controls only in the 40R group. Fetal and placental weights were reduced by undernutrition in all restricted groups. The greatest reductions were seen in the 40R and R+F groups (33%) and the least in the R+P group (16%). By birth the R+P group was intermediate between the control group and the remaining restricted groups. Neonatal mortalities also followed the same pattern and were restricted to the smallest neonates in each group; mean body weight of neonatal mortalities was 45·28 g (range 29·7–52·5 g, *n* 16).

Fibre numbers

Biceps brachii primary fibre number and mean S:P ratio were determined from 50 d fetal muscles and are shown in Fig. 1(*a* and *b*). Total muscle fibre numbers were counted in both 50 d fetuses and neonates. Results from fetuses and neonates could be combined since ANOVA tests revealed no significant difference in myofibre number between the two ages for any nutritional groups.

Primary fibre number did not differ between groups ($P > 0{\cdot}05$). However, mean S:P ratio was similar for control, R+P and R+C groups and significantly greater than those for 40R and R+F groups ($P < 0{\cdot}05$) by approximately 13·5%. Combined total fibre number data (Fig. 1(*c*)) demonstrate a similar pattern to that of mean S:P ratio, with total fibre number being reduced by 14–16% in the 40R and R+F groups ($P < 0{\cdot}05$).

DISCUSSION

In the present study it has been shown that a restricted maternal diet supplemented with either protein or carbohydrate was sufficient to maintain levels of secondary fibre hyperplasia (Fig. 1(*b*)) in the fetus at those of *ad lib.* controls, resulting in no reduction in *biceps brachii* fibre number (Fig. 1(*c*)). Primary fibre number is unaffected in any group

Table 2. *Effect of nutritional treatments on maternal weight gain, and on fetal, placental and neonatal weights in guinea-pigs**

(Values are means with their standard errors)

Dietary group...	Control		40R		R+P		R+C		R+F	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
50 d gestation										
Daily maternal wt gain (g)	5·6	1·3	0·2	1·8	3·4	0·8	1·8	1·4	−0·7	4·1
No. of dams	3		3		3		3		3	
No. of fetuses	9		13		13		10		12	
Term										
Daily maternal wt gain (g)	7.5[a]	1·1	0.7[b]	0·9	3.9[ab]	1·9	3·3[ab]	0·1	2·6[ab]	0·5
No. of dams	3		3		4		3		3	
No. of young	12		13		11		13		14	
Litter size: Median	3·5		4·5		3		4		4	
Range	2–5		3–5		2–5		2–6		2–6	
Fetal wt at 50 d (g)	36·6[a]	2·8	24·2[b]	1·8	30·6[b]	1·4	29·0[b]	1·5	24·6[b]	1·5
Percentage reduction	0·0		33·8		16·4		20·7		32·9	
Placental wt (g)	5·7[a]	0·4	3·6[b]	0·2	4·3[b]	0·2	4·5[b]	0·3	3·6[b]	0·2
Percentage reduction	0·0		37·6		24·9		21·3		36·5	
Birth wt (all) (g)	90·4[a]	4·6	51·1[c]	3·1	71·8[b]	3·7	59·4[c]	4·9	52·1[c]	3·6
Birth wt (live) (g)	90·4[a]	4·6	56·2[c]	3·4	73·3[b]	3·8	66·6[c]	5·1	67·5[c]	5·6
Percentage reduction	0·0		37·8		18·9		26·3		25·4	
Percentage mortality	0·0		46·15		9·10		30·86		46·15	

[a,b,c] Mean values with unlike superscript letters were significantly different ($P < 0·05$).

Control, *ad lib.* access to SG1 pellets (Grain Harvesters, Wingham, Kent); 40R, pair-fed with SG1 pellets at 60% of *ad lib.* intake; R+P, protein supplemented; R+C, carbohydrate supplemented; R+F, fat supplemented.

* For details of diets and procedures, see p. 174.

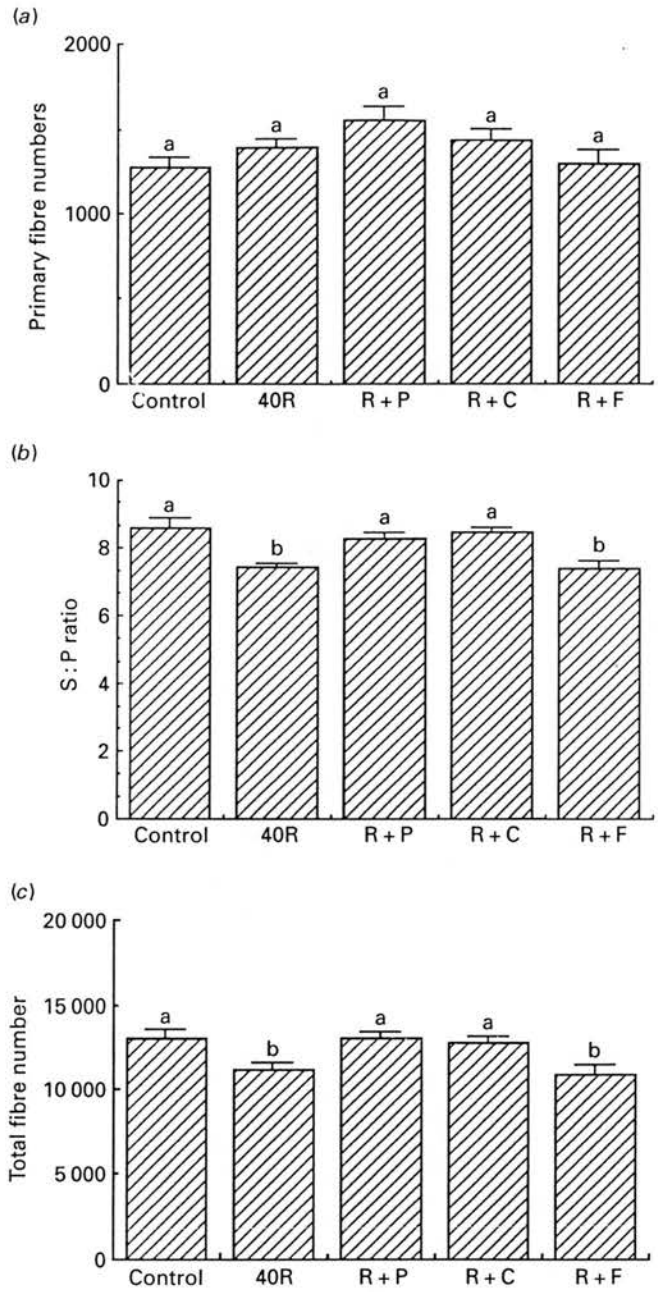

Fig. 1. Effect of nutritional treatments on mean *biceps brachii* myofibre numbers of guinea-pigs. (*a*) Primary fibre number, (*b*) secondary:primary fibre number (S:P) ratio, and (*c*) total myofibre number. Control, *ad lib.* access to SG1 pellets (Grain Harvesters, Wingham, Kent); 40R pair-fed with SG1 pellets at 60% of *ad lib.* intake; R+P, protein supplemented; R+C, carbohydrate supplemented; R+F, fat supplemented; for details of diets and procedures, see p. 174. Values are means with their standard errors represented by vertical bars. a,b, Mean values with unlike superscript letters were significantly different ($P < 0.05$).

(Fig. 1(*a*)). However, none of the restricted diets, despite supplementation, was able to maintain fetal growth at the same rate as that seen in the control *ad lib.*-fed fetuses (Table 2). The birth weights of the R+P protein-supplemented group were intermediate between those of the controls and other restricted groups. A high level of neonatal mortality was seen in the 40R (60%-restricted) and R+F (fat-supplemented) groups (Table 2), which was restricted to the smallest neonates. In general, it seems that a body weight of less than 50 g severely compromises the survival of the neonatal guinea-pig. A low birth weight tends to be associated with a reduced myofibre number (Handel & Stickland, 1987), therefore, it is likely that these animals possessed a low fibre number. Thus, it is likely that the mean total fibre numbers of the 40R and R+F groups are an overestimation of the true values. This would explain the reduction in fibre number of only 15% in these groups; reductions of 20% have been reported for this level of undernutrition (Ward & Stickland, 1991; Dwyer & Stickland, 1992). However, these groups still had a significant reduction in fibre number (Fig. 1(*c*)). Furthermore, Fig. 1(*b*) demonstrates a significant reduction in secondary fibre proliferation in the 40R and R+F groups only, determined at day 50 when all animals were included. Therefore, despite the 30% neonatal mortality in the R+C group (Table 2), it is likely that the estimate for total myofibre number in this group is accurate and that fibre development in only the 40R and R+F groups was affected by the nutritional treatment. The 9% neonatal mortality in the R+P group is within the normal preweaning rate found in the guinea-pig (Sutherland & Festing, 1986).

Placental weight was reduced also in all restricted fetuses at 50 d gestation (Table 2). This tended to be by a similar amount to fetal weight, except in the R+P group where the decrease in placental weight seemed to exceed the decrease in fetal weight (respectively 25 and 16%). This suggests that the placentas of protein-supplemented animals may be more efficient than those of other groups such that a heavier weight of fetus is supported per unit weight of placenta. Evidence from the pig suggests that maternal protein levels at the time of implantation may be important for the early establishment and growth of the placenta (Pond *et al.* 1969).

The important metabolic fuels in the fetus are glucose, amino acids, lactate and fatty acids (Jones & Rolph, 1985). The fetus has high plasma and tissue levels of amino acids which are actively transported over the placenta from the maternal circulation (Jones & Rolph, 1985). Amino acids may supply as much as 25% of the oxidative needs of the ovine fetus (Gresham *et al.* 1972). Thus, amino acids make an important contribution to fetal oxidative metabolism, and a decrease in glucose supply can cause a shift to increased amino acid degradation (Battaglia & Meschia, 1978). This suggests that under conditions of low maternal plasma glucose there may be an increased fetal dependence on amino acids for oxidative metabolism at the expense of protein accretion. This may explain why R+P fetuses were lighter than controls despite similar maternal protein intakes. R+P fetuses were, however, significantly heavier than those of the other nutritional groups; thus, some of the extra protein must have been available to the fetuses for tissue accretion.

Amino acids may also act as precursors for gluconeogenesis. The placenta is incapable of gluconeogenesis (Bossi & Greenberg, 1972); therefore, any newly synthesized glucose must be of fetal or maternal origin. The gluconeogenic enzymes are present in the guinea-pig liver from day 40 of gestation (Jones & Ashton, 1976), but gluconeogenesis itself does not take place until late gestation (Jones & Ashton, 1976; Faulkener & Jones, 1976). Although there is some evidence that undernutrition may lead to an early initiation of gluconeogenesis (Jones, 1982), endogenous production of glucose by the fetus is unlikely to affect fibre numbers since the very early stages of gestation, the first trimester, appear to be the critical period for determination of fibre numbers (Dwyer *et al.* 1993). However, there may have been some maternal synthesis of glucose.

Proteins may also be involved in the maintenance of fetal fibre numbers in an indirect manner by altering or maintaining the endocrine status of the fetus, particularly via insulin and the insulin-like growth factors (IGF). Plasma insulin is reduced in the porcine fetus when the sow is fed on a diet deficient in protein (Atinmo *et al.* 1976), and in children with kwashiorkor (Soliman *et al.* 1986). Secretion of insulin has been shown to be sensitive to dietary protein (Jepson *et al.* 1988). Protein restriction of pregnant rats causes a decrease in circulating somatomedin levels in both fetal and maternal serum (Pilistine *et al.* 1984). Protein restriction of young postnatal rats also causes a reduction in serum IGF-1 and liver and muscle IGF-1 mRNA (Moats-Staats *et al.* 1989; Yahya *et al.* 1990; Vandehaar *et al.* 1991). IGF-1 appears to be linearly related to protein levels when energy levels are kept constant (Dardevet *et al.* 1991). Therefore, IGF-1 levels appear to be maintained independently by carbohydrate and protein levels. Thus, both the levels of glucose and amino acids may act via an insulin pathway, involving insulin and the IGF, to affect fibre number.

The role of fats in fetal muscle development appear fairly minor since plasma levels of fatty acids in the fetus are usually low until late gestation (Jones & Rolph, 1985). In addition, higher levels of fat in the maternal diet seemed ineffective at preventing low birth weight and postnatal mortality (Table 2).

In conclusion, the hypothesis that the carbohydrate portion of the diet would be sufficient to maintain control numbers of muscle fibres was confirmed. However, the protein portion of the maternal diet was also found to be equally effective. The extra protein in the diet may have been used as a supply of amino acids for oxidative metabolism, as precursors for gluconeogenesis, and/or may have played a role in the maintenance of fetal endocrinology, such that fibre number development was not impaired.

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REFERENCES

Anugwa, F. O. I. & Pond, W. G. (1989). Reproduction and organ weights in rats fed high protein, restricted balanced diets or restricted nonprotein calories. *Nutrition Reports International* **40**, 879–892.

Atinmo, T., Baldijao, C., Pond, W. G. & Barnes, R. H. (1976). Maternal protein malnutrition during gestation alone and its effects on plasma insulin levels of the pregnant pig, its fetuses and the developing offspring. *Journal of Nutrition* **106**, 1647–1653.

Battaglia, F. C. & Meschia, G. (1978). Principle substrates of fetal metabolism. *Physiological Reviews* **58**, 499–527.

Bossi, E. & Greenberg, R. E. (1972). Sources of blood glucose in the rat fetus. *Pediatric Research* **6**, 765–772.

Cheek, D. B. & Hill, D. E. (1970). Muscle and liver cell growth: role of hormones and nutritional factors. *Federation Proceedings* **29**, 1503–1509.

Dardevet, D., Manini, M., Balage, M., Sornet, C. & Grizard, J. (1991). Influence of low- and high-protein diets on insulin and IGF-1 binding to skeletal muscle and liver in the growing rat. *British Journal of Nutrition* **65**, 47–60.

Dwyer, C. M., Madgwick, A. J. A., Ward, S. S. & Stickland, N.C. (1993). The effect of maternal undernutrition, imposed before or after the first trimester, on muscle fibre development in the guinea pig. *Journal of Anatomy* **183**, 200 Abstr.

Dwyer, C. M. & Stickland, N. C. (1992). Does the anatomical location of a muscle affect the influence of undernutrition on muscle fibre number? *Journal of Anatomy* **181**, 373–376.

Faulkener, A. & Jones, C. T. (1976). Metabolic concentrations in the liver of the adult and developing guinea pig and the control of glycolysis *in vivo*. *Archives of Biochemistry and Biophysics* **176**, 171–180.

Gresham, E. L., James, E. J., Raye, J. R., Battaglia, F. C., Makowski, E. L. & Meschia, G. (1972). Production and excretion of urea by the foetal lamb. *Pediatric Research* **50**, 372–379.

Guth, L. & Samaha, F. J. (1970). Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28**, 365–367.

Handel, S. E. & Stickland, N. C. (1987). Muscle cellularity and birth weight. *Animal Production* **44**, 311–317.

Jepson, M. M., Bates, P. C. & Millward, D. J. (1988). The role of insulin and thyroid hormones in the regulation of muscle growth and protein turnover in response to dietary protein in the rat. *British Journal of Nutrition* **59**, 397–415.

Jones, C. T. (1982). Comparative aspects of hepatic glucose metabolism during foetal development. *Biochemical Society Transactions* **9**, 375–376.

Jones, C. T. & Ashton, I. R. (1976). The appearance, properties and functions of gluconeogenic enzymes in the liver and kidney of the guinea pig during foetal and early neonatal development. *Archives of Biochemistry and Biophysics* **174**, 506–522.

Jones, C. T. & Rolph, T. P. (1985). Metabolism during foetal life: a functional assessment of metabolic development. *Physiological Reviews* **65**, 357–430.

Moats-Staats, B. M., Brady, J. L., Underwood, L. E. & D'Ercole, A. J. (1989). Dietary protein restriction in artificially reared neonatal rats causes a reduction in IGF-1 gene expression. *Endocrinology* **125**, 2368–2375.

Pilistine, S. J., Moses, A. C. & Munro, H. N. (1984). Placental lactogen administration reverses the effect of a low protein diet on maternal and foetal serum somatomedin levels in the pregnant rat. *Proceedings of the National Academy of Sciences, USA* **81**, 5853–5857.

Pond, W. G. (1973). Influence of maternal protein and energy nutrition during gestation on progeny performance in swine. *Journal of Animal Science* **36**, 175–182.

Pond, W. G., Maurer, R. R. & Klindt, J. (1991). Fetal organ response to maternal protein deprivation during pregnancy in swine. *Journal of Nutrition* **121**, 504–509.

Pond, W. G., Strachan, D. N., Sinha, Y. N., Walker, E. F., Dunn, J. A. & Barnes, R. H. (1969). Effect of protein deprivation of swine during all or part of gestation on birth weight, postnatal growth rate and nucleic acid content of brain and muscle of progeny. *Journal of Nutrition* **99**, 61–67.

Pond, W. G., Wagner, W. C., Dunn, J. A. & Walker, E. F. (1968). Reproduction and early post natal growth of progeny in swine fed a protein-free diet during gestation. *Journal of Nutrition* **94**, 309–316.

Pond, W. G. & Wu, J. F. (1981). Mature body size and life span of male and female progeny of primiparous rats fed a low protein or adequate diet throughout pregnancy. *Journal of Nutrition* **111**, 1949–1954.

Pond, W. G., Yen, J.-T. & Mersmann, H. J. (1987). Effect of severe dietary protein, non-protein calories or feed restriction during gestation on postnatal growth of progeny in swine. *Growth* **51**, 355–371.

Pond, W. G., Yen, J.-T., Mersmann, H. J. & Maurer, R. R. (1990). Reduced mature size in progeny of swine severely restricted in protein intake during pregnancy. *Growth, Development and Aging* **54**, 77–84.

Soliman, A. T., Hassan Abd. el Hadi I., Aref, M. K., Hintz, R. L., Rosenfeld, F. G. & Rogol, A. D. (1986). Serum IGF-I and IGF-II concentrations and growth hormone and insulin responses to arginine infusion in children with protein-energy malnutrition before and after nutritional rehabilitation. *Pediatric Research* **20**, 1122–1130.

Sutherland, S. D. & Festing, M. F. W. (1986). The guinea pig. In *The UFAW Handbook on the Care and Management of Laboratory Animals*, pp. 393–410 [T. B. Poole, editor]. London: Longman Scientific & Technical.

Vandehaar, M. J., Moats-Staats, B. M., Davenport, M. L., Walker, J. L., Ketelslegers, J.-M., Sharma, B. K. & Underwood, L. E. (1991). Reduced serum concentrations of IGF-1 in protein-restricted growing rats are accompanied by reduced IGF-1 mRNA levels in liver and skeletal muscles. *Journal of Endocrinology* **130**, 305–312.

Ward, S. S. & Stickland, N. C. (1991). Why are fast and slow muscles differentially affected during prenatal undernutrition? *Muscle and Nerve* **14**, 259–267.

Winick, M. (1970). Nutrition and nerve cell growth. *Federation Proceedings* **29**, 1510–1515.

Yahya, Z. A. H., Bates, P. C. & Millward, D. J. (1990). Responses to protein deficiency of plasma and tissue IGF-1 levels and proteoglycan synthesis rates in rat skeletal muscle and bone. *Journal of Endocrinology* **127**, 497–503.

Young, V. R. & Alexis, S. D. (1968). In vitro activity of ribosomes and RNA content of skeletal muscle in young rats fed adequate or low protein. *Journal of Nutrition* **96**, 255–262.

Zeman, F. J. & Stanbrough, E. C. (1969). Effect of maternal protein deficiency on cellular development in the foetal rat. *Journal of Nutrition* **99**, 274–282.

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The Influence of Maternal Nutrition on Muscle Fiber Number Development in the Porcine Fetus and on Subsequent Postnatal Growth[1]

Catherine M. Dwyer*,[2], Neil C. Stickland*, and John M. Fletcher†

*Department of Veterinary Basic Sciences, Royal Veterinary College, London NW1 0TU, U.K. and †Unilever Research, Colworth House, Sharnbrook, Beds. MK44 1LQ, U.K.

STRACT: In the pig, undernutrition in utero ses low birth weight, a decrease in muscle fiber ıber, and a reduction in postnatal growth rate. The ct on fiber number is mediated via a reduced ndary fiber population. Within a litter of pigs, ter-weight pigs have probably suffered some deficit ıuscle fiber number. In an attempt to improve the ıber of fibers in the lighter-weight pig fetuses, four ernal feeding regimens were used, one serving as control. Maternal feed intake was doubled for one ıree time periods during pregnancy: 1) d 25 to 50) immediately before fiber hyperplasia; 2) d 50 to HL) during fiber hyperplasia; or 3) d 25 to 80 ') covering both developmental events. Controls e fed at levels routinely used for pregnant sows on farm. Sows farrowed normally and pig birth ghts were recorded. Estimates were made of total fiber number, total primary fiber number, and n secondary:primary fiber number ratio (S:P) for the semitendinosus of each pig at 5 wk postnatal or 80 kg (HT and two control litters only). The progeny of all supplemented sows had a significantly greater mean S:P ratio ($P < .05$), and the HE pigs tended to have a greater number of muscle fibers than control pigs (403,840 ± 8,197 vs 370,970 ± 12,720). Postnatal growth rate to 80 kg was also investigated for the HT group of pigs. The HT pigs had a faster growth rate from d 70 to slaughter at 80 kg (924.4 ± 18.75 g/d vs 840.1 ± 17.48 g/d, $P = .017$) and an increased gain:feed ratio (.433 ± .011 vs .401 ± .007, $P = .025$) compared with controls. These results suggest that the mean number of secondary fibers formed in a litter of pigs can be improved by increasing maternal feed intake during d 25 to 50 of gestation. This may have additional benefits in terms of improved growth rate and growth efficiency in the latter stages of pig growth to 80 kg.

Key Words: Pigs, Nutrition, Fetus, Myofiber, Growth

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Introduction

Iuscle fiber number is an important determinant ıuscle mass in the pig (Miller et al., 1975), and, efore, is of importance in the rearing of animals commercial purposes. Muscle fiber hyperplasia is plete by d 90 of gestation (Wigmore and Stick-., 1983) and is made up of contributions from ıary and secondary fibers. Primary fibers develop , by the rapid fusion of primary myoblasts, wed by a longer period of secondary fiber develop-t on the surface of the primary fibers (Kelly and Zacks, 1969; Duxson and Usson, 1989). Primary fiber numbers are resistant to environmental influences, but the number of secondary fibers that develop is susceptible to many factors, including nutrition (Wigmore and Stickland, 1983), and is responsible for most of the variation in fiber number seen within litters (Dwyer and Stickland, 1991). Factors affecting the development of secondary fibers occur in the prenatal period and are permanent thereafter.

Litters of pigs tend to show a wide distribution of birth weights, growth rates, and muscle fiber numbers (Handel and Stickland, 1987; Dwyer and Stickland, 1991). This variation has been shown to be due to in utero undernutrition (Wigmore and Stickland, 1983; Handel and Stickland, 1987), termed "natural undernutrition," which causes a reduction in muscle cellularity in the smallest pigs by affecting the secondary fiber population.

This study was designed to test the hypothesis that increasing sow feed intake at specific times during

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To whom correspondence should be addressed.

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station would result in an increase in muscle fiber mber in the fetuses by affecting the developing pulation of secondary fibers. Secondary fiber hyper-sia begins at approximately d 50 of gestation in the (Wigmore and Stickland, 1983) and continues til d 85 to 90. Three time periods were examined in s study: up to the onset of secondary fiber perplasia (gestational d 25 to 50), during fiber perplasia (d 50 to 80), and covering both time riods (d 25 to 80). If it is assumed that the largest s in a litter had achieved their maximum potential fiber development, the improvement in fiber mber of those pigs that would have formed the ver birth weight group should also lead to narrow-in the distribution of pig muscle fiber numbers.

Materials and Methods

Twenty-three Large White × Landrace pregnant vs (weight range at mating 164 to 194 kg, mean = 6 kg), in their third parity, were mated and intained under identical commercial conditions til d 20. Boars were randomly selected from a pool three. At d 20 of gestation, sows were randomly signed to a nutritional treatment. All sows were fed tandard commercial sow feed (5% oil, 17% protein, % fiber, 12.5 MJ of energy/kg of feed) throughout experiment. Experimental groups differed in the antity of feed during specific periods of gestation. ntrol sows (n = 7) were fed 2.5 kg/d throughout the perimental period (d 21 to 90 of pregnancy), as ommended by the feed manufacturer. There were ee additional experimental groups: 1) **HE** sows (n) were fed 5 kg/d from d 25 to 50; 2) **HL** sows (n = were fed 5 kg/d from d 50 to 80; and 3) **HT** sows (n) were fed 5 kg/d from d 25 to 80. Target intakes re achieved over 5 d by .5-kg steps up or down in d offered (e.g., HE sows received 3 kg on d 21 and 5 by d 25). Sows returned to control levels of dietary ake (2.5 kg/d) after the experimental period. Feed s offered in two equal-sized meals daily. Sows were intained in large, well-ventilated straw yards (15 20°C, 12 h light/d) and entered individual stalls at d times. Five days before farrowing sows were ced in farrowing crates in individual pens.

In the pig, secondary myofiber hyperplasia begins approximately d 50 of gestation (Wigmore and ckland, 1983) and is thought to be essentially nplete by d 85 to 90. The time periods were selected coincide with the period of maximum secondary er hyperplasia (HL), or immediately prior to the set of hyperplasia (HE). The HT treatment was signed to evaluate the overall effect of elevated trient intake during both time periods. From d 80 of tation to farrowing all sows were fed and handled ntically (i.e., 2.5 kg/d from d 80 to 90, 3.0 kg/d from 90 to 112, and 2.3 kg/d until farrowing). Feeding ring lactation was also similar for all sows (i.e., 4 kg/d plus 200 g per pig suckled). All feed was readily consumed. Pigs were weighed at birth and at weaning. The litters from the HE and HL groups and five litters of control pigs were killed at 5 wk of age by an overdose injection of pentobarbitone sodium (Euthesate) into the heart. Pigs were killed at 5 wk of age because, in the pig, maturation of muscle fiber type differentiation by myosin ATPase staining is not seen until 4 wk postnatal (Cooper et al., 1970; Handel, 1984). The two remaining control litters and the five HT litters were grown to approximately 80 kg in weight, to assess the effect of maternal nutrition on postnatal growth to commercial slaughter weight. Pigs were housed in small groups of three to four pigs of similar weight in straw pens at a density of one pig per square meter. Pigs were fed a weaning diet (8% oil, 21.5% protein, 2% fiber, 15.4 MJ/kg of diet) to 5 kg in weight, a growing diet (5.5% oil, 22% protein, 3% fiber, 14.4 MJ/kg of diet) to 26 kg, and a finishing diet (4.5% oil, 20% protein, 3.5% fiber, 13.9 MJ/kg of diet) to slaughter weight of 80 kg. Pigs were weighed at 1-mo intervals after weaning. Boars were raised as intact males. Pigs were killed at approximately 130 d of age by standard abattoir procedures.

For all pigs (n = 224) the left semitendinosus muscle was removed, trimmed of fat and connective tissues, and weighed. A complete cross-section from the center of the muscle, of approximately 5 mm in depth, was frozen in dichlorodifluoromethane cooled to freezing (−158°C) in liquid nitrogen. Sections of 10-μm thickness were cut at −25°C in a cryostat and stained for myosin adenosine triphosphatase (ATPase) activity, using the method of Guth and Samaha (1970). Estimations were made from these sections for the total muscle fiber number, total primary fiber number, and the mean ratio of secondary fibers: primary fibers (**S:P ratio**) as previously described (Dwyer and Stickland, 1991). Briefly, the total cross-sectional area of the muscle was measured, then estimations of total muscle fiber number and primary fiber number were calculated after counting between 3 and 5% of the total number in randomly selected areas, using a Seescan image analysis system (Seescan plc, Cambridge, U.K.). Mature pigs exhibit a unique arrangement of muscle fibers, with central groups of slow fibers surrounded by large numbers of fast fibers. From previous studies (Wigmore and Stickland, 1983) it is known that one of the central slow fibers in each cluster was a primary myofiber, whereas all others developed as secondary fibers. This means, therefore, that the number of primary and secondary fibers that formed during development prenatally can be determined in the postnatal pig. The total number of secondary fibers is dependent on the number of primary fibers because they form on the surface of the primary fibers during muscle development (Kelly and Zacks, 1969). The S:P ratio is, therefore, used in this study because it provides an index of the effect of nutrition on the susceptible

Table 1. Effect of level of sow feed intake on litter size, mean pig birth weight, and mean weaning weight (21 days)[a]

n	Level of feed					
	Control	HE[b]	HL[c]	HT[d]	SEM[e]	Probability
of litters	7	5	6	5	—	—
of pigs	67	49	61	47	—	—
born/sow	11.4[f]	14.6	11.2	11.8	.57	NS[g]
live/sow	10.0[f]	12.8	10.3	10.8	2.63	NS
weaned/sow	9.6[f]	9.8	10.2	9.6	2.20	NS
er wt (d 0), kg	15.55[f]	17.74	15.41	15.93	1.68	NS
h wt/pig, kg	1.57[f]	1.41	1.50	1.51	.18	NS
er wt (d 21), kg	58.5	64.4	66.1	62.6	13.44	NS
aning wt/pig, kg	6.16	6.66	6.50	6.60	.80	NS

[a]ANOVA tests.
[b]HE = High intake d 25 to 55.
[c]HL = High intake d 50 to 80.
[d]HT = High intake d 25 to 80.
[e]SEM = pooled standard error.
[f]Based on five litters of pigs only, n = 48.
[g]NS = not significant ($P > .05$).

nponent (the secondary fibers), while excluding the luence of the genetic component (the primary ers).

Nutritional treatment groups were compared by NOVA and Newman-Keuls tests at 5% probability el, following a significant F-test, using sows as the perimental unit to compensate for genetic effects. In dition, the distribution of muscle fiber numbers for pigs within a treatment group was investigated, ng Bartlett's test for the homogeneity of variance, cause, as suggested in the introduction, improved v nutrition may lead to a narrowing of the tribution of fiber numbers. The growth rates and iciencies of the 80-kg pigs (control and HT pigs) re compared by t-tests, using the pen as the perimental unit.

Results and Discussion

Weight and Survival

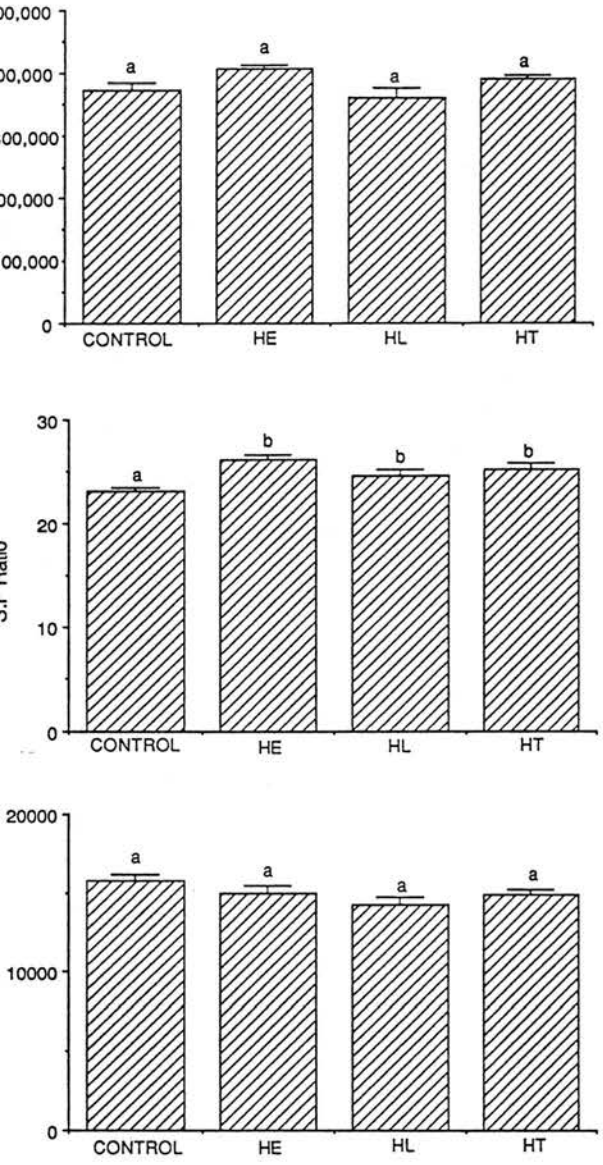
The numbers of pigs born and surviving and pig th and weaning weights are given in Table 1. Two ers of control pigs were not weighed at birth; refore, the first comparison involving every animal at weaning. Nutritional treatments had no signifi- nt effect on litter size or on pig mortality.

Average pig birth weights were not significantly ected by the nutritional treatments (Table 1). All tritional treatments ceased before the third trimes- and sows were fed equally during the last third of tation and during lactation. In the rat, restricting ternal intake in the second half of pregnancy only s found to have an effect on pup birth weight nderson et al., 1980). Similarly, in the pig, ternal feed intake was found to affect birth weight y after d 80 of gestation (Noblet et al., 1985). Maternal feed intake during the last one-half to one-third of gestation, therefore, seems to have the greatest effect on the birth weight of offspring. The sows used in this study were fed similarly over the last half of gestation, and therefore it is not surprising that mean birth weights of the nutritional groups were similar.

Muscle Fiber Number Data

The mean data for total fiber number, total primary fiber number, and S:P ratio for each nutritional group are given in Figure 1. Muscle cellularity data from 5-wk-old pigs and 130-d-old pigs can be combined because total muscle fiber number is determined before birth in the pig (Wigmore and Stickland, 1983). It has been suggested, for the porcine sartorius muscle (Swatland and Cassens, 1972), that intrafascicularly terminating fibers may grow into the plane of section, such that the number of muscle fibers, determined by the method used here, show an age- and weight-dependent increase (Swatland, 1976). However, in the semitendinosus muscle, no evidence has been found of intrafascicularly terminating fibers (Wigmore and Stickland, 1983). Furthermore, muscle fiber number remains constant for this muscle with an increase in age and live weight (Handel and Stickland, 1987). Finally, there was no significant difference (ANOVA) in muscle cellularity between 5-wk control pigs and 130-d control pigs in the present study. For these reasons it was considered valid to combine data from pigs of different ages, and that calculated fiber number differences represent real differences between pigs.

When compared by ANOVA and Newman-Keuls tests there were no significant differences between nutritional treatments in total muscle fiber number (Figure 1a). However, total fiber number tended to be



ure 1. Means (± SE) of semitendinosus fiber er of pigs for each nutritional group of sows: (a) iber number (C = 370,970 ± 12,720, HE = 0 ± 8,197, HL = 360,430 ± 15,088, HT = 389,950 57); (b) mean secondary:primary fiber number C = 23.17 ± .37, HE = 26.16 ± .50, HL = 24.60 ± T = 25.20 ± .57); and (c) primary fiber number (C 805 ± 409, HE = 14,960 ± 484, HL = 14,270 ± IT = 14,908 ± 275). Significance ($P < .05$) was nined by Newman-Keuls ANOVA and is indicated fering letters. Control = sows fed 2.5 kg/d, d 21 to tation; HE = sows fed 5 kg/d, d 25 to 50; HL = ed 5 kg/d, d 50 to 80; HT = sows fed 5 kg/d, d 25

in the HE and HT groups (HE = 403,840 ± HT = 389,940 ± 6,067; C = 370,970 ± 12,720). ugh none of the high intake groups differed from

another in S:P ratio, the HE, HL, and HT groups had larger mean ratios compared with the control group (HE = 26.16 ± .5; HL = 24.60 ± .55; HT = 25.20 ± .57; C = 23.17 ± .37; $P < .05$, Figure 1b) by 12.9, 6.2, and 8.8%, respectively. There was no significant difference between nutritional treatments in primary fiber number (Figure 1c).

In pigs the distribution of fetal weights in a litter suggests that the smaller littermates have experienced some degree of undernutrition in utero in sows fed at commercial levels (Wigmore and Stickland, 1983). Low birth weight pigs have smaller and lighter muscles with less DNA (Widdowson, 1971; Campbell and Dunkin, 1982) and a low muscle fiber number (Powell and Aberle, 1980, 1981). Examination of the semitendinosus muscle from large and small pig fetus littermates and in postnatal animals has revealed that muscles from smaller animals have a reduced total fiber number caused by a reduced secondary fiber population (Wigmore and Stickland, 1983; Handel and Stickland, 1987). This study extends these conclusions by suggesting that it may be possible to increase the number of secondary fibers in the developing pig fetuses by improving the nutritional status of the sow.

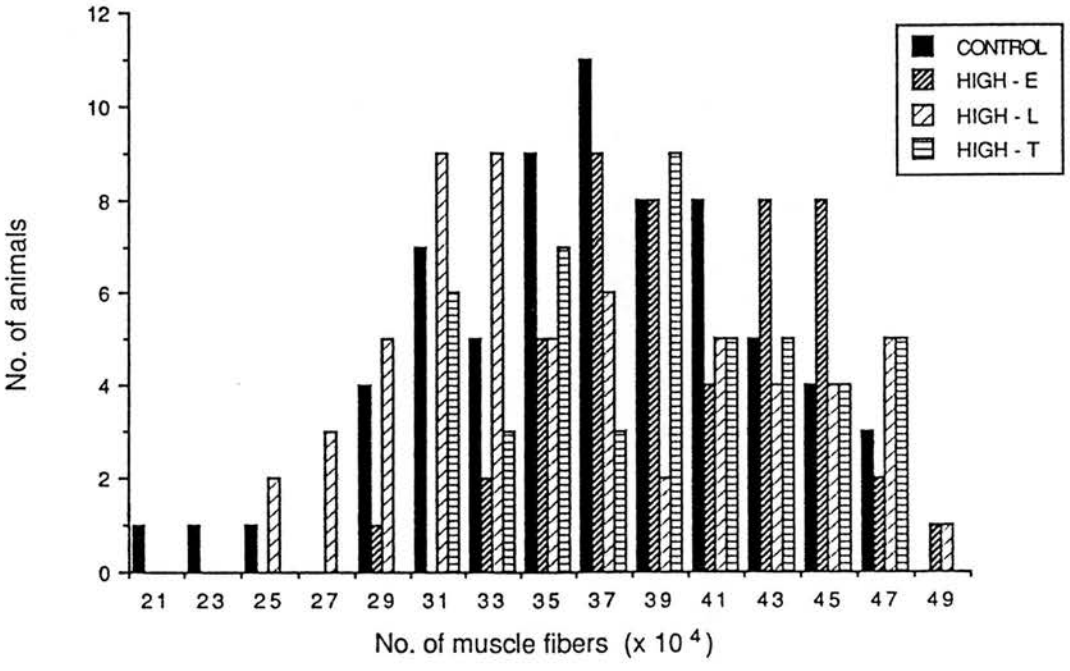
The two feed groups that included the early time period (d 25 to 55; i.e., HE and HT) seemed to effect the greatest increase in mean number of secondary fibers formed per primary fiber. Increasing feed intake during fiber hyperplasia, the HL group, produced only a small increase in secondary fibers. Increasing the time period of extra intake (HT) gave no increased benefit in terms of an improved secondary fiber population over that achieved in the HE group. This suggests, therefore, that nutrition level in the period immediately before the appearance of secondary myotubes may be more important than that during the actual appearance of myofibers. A recent study in the guinea pig (Madgwick, 1991) has suggested that the cause of a low secondary fiber population is reduced nuclear proliferation in the undernourished animals. Early nutrition may, therefore, be crucial for the proliferation of presumptive secondary myoblasts, creating a greater potential for secondary fiber formation.

In this study, as in many others, the primary fiber population was unaltered by nutritional manipulations. This may be because they form relatively early in gestation, being present at d 38 of gestation in the pig (Wigmore and Stickland, 1983), when the litter is making a negligible nutritional demand on the sow. In addition, primary fibers form a relatively small proportion of the total muscle fiber population (i.e., less than 5% in the pig semitendinosus [personal observation]). Despite the small direct contribution made by the primary fiber population to the total fiber number, primary fiber number does have an important indirect influence because it forms the framework on which the secondary fibers form (Kelly and Zacks,

9; Duxson and Usson, 1989). The influence of
nary fiber number variations seems to be sufficient
liminate any significant effect on total muscle fiber
ıber caused by an increase in the S:P ratio.
vious work (Dwyer and Stickland, 1991) suggests
total fiber number and primary fiber number
v a much greater variation between litters than
S:P ratio. With a larger sample size than the
ent experiment, variations in primary fiber num-
would be reduced, and the increased production of
ndary fibers would cause an increase in total
cle fiber number.
has been suggested that a further consequence of
easing the sow feed intake in gestation would be to
ease the distribution of muscle fiber numbers
in a litter, by increasing the fiber numbers of the
itionally disadvantaged pigs without affecting the
er pigs. Within the same treatment group the
ance of muscle fiber numbers about the mean for
s was homogeneous (Bartlett's tests). However,
n treatment groups were compared, the distribu-
of HE and HT group fiber numbers were
ificantly smaller than in the HL group (HE, $P <$
HT, $P < .05$; Bartlett's test for the homogeneity of
ance), and the variance in the HE group was also
ller than that in the control group ($P < .05$). For
e tests data from all pigs were used because the
othesis was that the distribution within the litter
ld be affected. The distribution of muscle fiber
bers is shown in Figure 2 and demonstrates that
groups had representatives in the higher fiber number categories. It therefore seems that the original assumption, that the largest pigs had achieved their maximum growth potential in terms of fiber number, was valid. Comparison of the largest pigs from each sow by ANOVA demonstrated that there was no significant difference between groups (C = 451,671 ± 10,971; HE = 467,480 ± 9,127; HL = 453,800 ± 11,933; HT = 465,740 ± 4,298). However, the smallest fiber number classes were seen only in pigs from the control group, and in a few from the HL group (Figure 2). Comparison of the smallest pigs from each sow by ANOVA demonstrated a significant difference between groups (C = 296,390 ± 18,204; HE = 343,740 ± 16,700; HL = 277,050 ± 10,630; HT = 315,560 ± 5,451; $P <$ .05). This suggests that, although the number of fibers cannot be increased above a maximum by increasing maternal nutrition, it is possible to improve muscle fiber number at the lower range, and this leads to a narrower distribution of fiber numbers.

Growth Rate Data

The growth rates of the HT pigs (47 pigs, n = 12 pen) and of the two control litters (19 pigs, n = 5 pens) could be divided into three main phases, as previously described (i.e., birth to weaning, weaning to 70 d, and 70 d to slaughter; Dwyer et al., 1993). There were no significant differences between the two groups in mean growth rates and mean gain/feed ratio until d 70 (Table 2). Thereafter, the growth rate of the HT group was significantly greater than that of



gure 2. Distribution of semitendinosus fiber numbers for all pigs and each nutritional group. Control = sows
2.5 kg/d, d 21 to 90 gestation; High-E = sows fed 5 kg/d, d 25 to 50; High-L = sows fed 5 kg/d, d 50 to 80; High-
sows fed 5 kg/d, d 25 to 80.

Table 2. Average daily gain (ADG) and gain/feed ratios of 130-day-old pigs for linear growth phases[a]

Item	Level of feed		Probability
	Control	HT[b]	
ADG			
Weaning to d 70, g/d	467.4 ± 18.35	465.4 ± 14.59	NS[c]
Days 70 to 130, g/d	840.1 ± 17.48	924.4 ± 18.75	.017
Gain/feed ratio, g/g	.401 ± .007	.433 ± .011	.025

[a]Student's *t*-tests.
[b]HT = High intake at d 25 to 80.
[c]NS = not significant ($P > .05$).

ontrol litters (10%, $P = .017$, df = 15). This study both intact boars and gilts. There were more than gilts in both treatment groups (1.4:1 in ols; 1.6:1 in HT). Sex is known to have no effect uscle fiber number in mice (Rowe and Goldspink,) and did not affect muscle fiber number in pigs is study. Gilts grew more slowly than boars in control (11.2%, $P < .005$) and HT groups (6.8%, 05). However, this difference could not account fferences between treatment groups because HT had a higher growth rate than control boars 52 ± 18.24 vs 879.5 ± 17.26; $P < .01$), and HT grew faster than control gilts (889.33 ± 13.01 vs 0 ± 13.75; $P < .001$). The mean gain/feed ratio of igs was also increased (8%, $P = .025$, df = 15) g this period. It, therefore, seems that the effects enatal nutrition are felt in the later growth e. This period has been identified as the time growth is determined by the pig's own genotype nn et al., 1953). In addition, this growth phase is ficantly correlated with muscle fiber number of semitendinosus (Dwyer et al., 1993), unlike r growth, which is correlated with birth weight. ilar, but opposite, effect on growth rate has been by restricting maternal intake in pregnant sows, ng to a reduction in progeny ADG from wk 10 atal onward (Pond et al., 1985; Pond and mann, 1988). Rat pups restricted by maternal rnutrition had a lower mature weight than ols (Pond and Wu, 1981) due to a lower growth regardless of postnatal feed level (Cha and Oh,). Under conditions of natural undernutrition er littermates have lower live weights at a given han their larger littermates (Handel and Stick- 1988). Runt pigs also grow more slowly and less ently than their larger littermates (Powell and e, 1980) and are smaller at maturity (Wid- on, 1971). A constant feature of in utero under- tion, whether natural or caused by maternal feed ction, therefore, seems to be a reduction in h rate, growth efficiency, and mature size. In study it has been demonstrated that improved rnal nutrition during pregnancy can cause an improved growth rate and efficiency of gain in the postnatal progeny. Mean S:P ratio has been shown to be correlated with growth rate (Dwyer et al., 1993), and in the present study an increased maternal intake caused an increased S:P ratio (Figure 1b) and an improved growth rate (Table 2). It is possible that the increase in the number of secondary fibers and the increased growth rate may be independent consequences of increasing maternal feed intake, perhaps via an effect on growth factor levels. Fast-growing strains of pigs have a high myofiber number (Ezekwe and Martin, 1975) but also tend to have higher serum insulin-like growth factor I levels than do slow-growing pigs (Buonomo et al., 1987). Thus, it is impossible to distinguish the causal factor resulting in an increased postnatal growth rate.

In conclusion, this study has demonstrated that an increased maternal feed intake, for approximately 30 d, timed to occur immediately before muscle fiber hyperplasia results in an increase of 9 to 13% in the production of secondary myofibers. The increased muscle fiber number has a significant effect on growth rate and feed conversion efficiency in the latter stages of pig growth to 80 kg. These results suggest, therefore, that increasing sow feed intake in early pregnancy may improve the mean postnatal growth rate of the litter in the later growth phases to slaughter.

Implications

This work has demonstrated that increased maternal nutrition during early to midgestation in the pig can lead to an increased production of secondary myofibers in the fetus. Pigs resulting from pregnancies during which sows received an increased amount of feed during early gestation had faster growth rates and greater gain/feed ratios than control pigs. This suggests a possible strategy to increase muscle percentage in pigs and to improve growth rate to slaughter that needs to be assessed in terms of economic viability.

Literature Cited

derson, G. D., R. A. Ahokas, J. Lipshitz, and P. V. Dilts. 1980. Effect of maternal dietary restriction during pregnancy on maternal weight gain and foetal birth weight in the rat. J. Nutr. 110:883.

ınn, C. T., G. N. Baker, and L. E. Hanson. 1953. Heritability of gain in different growth periods in swine. J. Anim. Sci. 12:39.

onomo, F. C., T. J. Lauterio, C. A. Baile, and D. R. Campion. 1987. Determination of insulin-like growth factor-1 (IGF-I) and IGF binding protein levels in swine. Domest. Anim. Endocrinol. 4:23.

mpbell, R. G., and A. C. Dunkin. 1982. The effects of birth weight and level of feeding in early life on growth and development of muscle and adipose tissue in the young pig. Anim. Prod. 35:185.

a, C.-J., and W. Oh. 1986. Growth and fatty acid metabolism in experimental intrauterine growth retardation: Effect of postnatal nutrition in the rat. J. Nutr. 116:1080.

oper, C. C., R. G. Cassens, L. L. Kastenschmidt, and E. J. Briskey. 1970. Histochemical characterization of muscle differentiation. Dev. Biol. 23:169.

xson, M. J., and Y. Usson. 1989. Cellular insertion of primary and secondary myotubes in embryonic rat muscles. Development 107:243.

yer, C. M., J. M. Fletcher, and N. C. Stickland. 1993. Muscle cellularity and postnatal growth in the pig. J. Anim. Sci. 71: 3339.

yer, C. M., and N. C. Stickland. 1991. Sources of variation in myofibre number within and between litters of pigs. Anim. Prod. 52:527.

ekwe, M. O., and R. J. Martin. 1975. Cellular characteristics of skeletal muscles in selected strains of pigs and mice and the unselected controls. Growth 39:95.

th, L., and F. J. Samaha. 1970. Research note: procedure for the histochemical demonstration of actomyosin ATPase. Exp. Neurol. 28:365.

ndel, S. E. 1984. Effects of low birth weight on postnatal development of skeletal muscle in the pig. Ph.D. Dissertation. University of Edinburgh, U.K.

ndel, S. E., and N. C. Stickland. 1987. Muscle cellularity and birth weight. Anim. Prod. 44:311.

Handel, S. E., and N. C. Stickland. 1988. Catch-up growth in pigs: A relationship with muscle cellularity. Anim. Prod. 47:291.

Kelly, A. M., and S. I. Zacks. 1969. The histogenesis of rat intercostal muscle. J. Cell. Biol. 42:135.

Madgwick, A. J. 1991. The effect of maternal undernutrition on foetal myogenesis and development in the guinea pig (*Cavia porcellus*). Ph.D. Dissertation. University of London.

Miller, L. R., V. A. Garwood, and M. D. Judge. 1975. Factors affecting porcine muscle fiber type, diameter and number. J. Anim. Sci. 41:66.

Noblet, J., W. H. Close, R. P. Heavens, and D. Brown. 1985. Studies on the energy metabolism of the pregnant sow. 1. Uterus and mammary tissue development. Br. J. Nutr. 53:251.

Pond, W. G., and H. J. Mersmann. 1988. Comparative responses of lean or genetically obese swine and their progeny to severe feed restriction during gestation. J. Nutr. 118:1223.

Pond, W. G., H. J. Mersmann, and J.-T. Yen. 1985. Severe feed restriction of pregnant swine and rats: Effect on postweaning growth and body composition of progeny. J. Nutr. 115:179.

Pond, W. G., and J. F. Wu. 1981. Mature body size and life span of male and female progeny of primiparous rats fed a low protein or adequate diet throughout the pregnancy. J. Nutr. 111:1949.

Powell, S. E., and E. D. Aberle. 1980. Effects of birth weight on growth and carcass composition of swine. J. Anim. Sci. 50:860.

Powell, S. E., and E. D. Aberle. 1981. Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. J. Anim. Sci. 52:748.

Rowe, R. W., and G. Goldspink. 1969. Muscle fibre growth in five different muscles in both sexes of mice. J. Anat. 104:519.

Stickland, N. C., and S. E. Handel. 1986. The numbers and types of muscle fibres in large and small breeds of pigs. J. Anat. 147: 181.

Swatland, H. J. 1976. Effect of growth and plane of nutrition on apparent muscle fiber numbers in the pig. Growth 40:285.

Swatland, H. J., and R. G. Cassens. 1972. Muscle growth: the problem of muscle fibers with an intrafascicular termination. J. Anim. Sci. 35:336.

Widdowson, E. M. 1971. Intrauterine growth retardation in the pig. Biol. Neonate 19:329.

Wigmore, P. M., and N. C. Stickland. 1983. Muscle development in large and small pig foetuses. J. Anat. 137:235.

Reprod. Fertil. Dev., 1995, 7, 1285–92

Effect of Maternal Undernutrition in Early Gestation on the Development of Fetal Myofibres in the Guinea-pig

Catherine M. Dwyer[A], Anthony J. A. Madgwick, Stephanie S. Ward and Neil C. Stickland

Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, England.

[A] *Present address: SAC, Genetics and Behavioural Sciences Department, Penicuik EH26 0QE, Scotland.*

Abstract. A 40% restriction in maternal feed intake throughout gestation in the guinea-pig results in a 35% reduction in fetal body weight at term and a 20–25% reduction in muscle fibre number. To investigate the effect of maternal undernutrition in early gestation, four nutritional treatments were used: controls—pregnant females fed ad libitum throughout gestation; TR—fed 60% ad libitum intake throughout gestation; ER—fed 60% ad libitum for the first third of gestation (until Day 25), then ad libitum to term; LR—fed ad libitum for the first 25 days, then 60% of ad libitum to term. The LR group were complicated by a high degree of fetal resorption and early littering of viable litters. The biceps brachii and soleus muscles were removed from neonates and total muscle fibre numbers determined. In a second experiment a further 8 pregnant guinea-pigs were fed 60% ad libitum until Day 15 of gestation only, and then rehabilitated onto an ad libitum diet (VER). Of these, 5 guinea-pigs were killed at term and the remaining 3 at 45 days gestation. Fetuses and placentae were obtained from all VER animals and compared with TR and controls of a similar age.

Body weights were reduced in all restricted groups at term when compared with controls ($P < 0{\cdot}05$) by 12, 40 and 50% for VER = ER, TR and LR groups, respectively. Biceps fibre number was reduced ($P < 0{\cdot}05$) in ER, TR and LR groups by 28, 20 and 25%, respectively, but was not affected in the VER group. Soleus fibre number was not significantly affected by any nutritional treatment. Restriction for 15 days in early gestation caused a significant 20% increase in fetal weight at 45 days' gestation compared with controls, but muscle and placental weights were not affected. Analysis of placental components at Days 45 and 65 suggested that underfeeding in early gestation and subsequent refeeding caused some placental adaptations to increase the exchange-surface area.

A short period of maternal undernutrition in the first third of gestation alone (ER), therefore, resulted in a biceps brachii fibre number deficit similar to that caused by restriction throughout gestation only if the period of restriction extended as far as Day 25. Furthermore, fetal weight at term was impaired by short-term nutritional restriction in early gestation. Restriction in the last two-thirds of gestation, following an ad libitum diet in the first third, caused a reduction in biceps fibre number and had a severe effect on the maintenance of pregnancy. It is probable that undernutrition in early gestation had an indirect effect on muscle fibre number by affecting the development of the placenta. This could be avoided by nutritional rehabilitation before Day 25 of gestation, but appeared to be permanent thereafter. Undernutrition after Day 25 may have had a direct effect on the development of secondary fibres.

Introduction

The increase in muscle fibre numbers is an early developmental event, occurring before birth in precocious species, and is complete by Day 50 of gestation in the guinea-pig (term is 68 days). Primary myotubes are first seen in the fetal guinea-pig at gestational Day 30, and secondary myotubes start to appear between Days 30 and 35 (Ward and Stickland 1991). Restriction of maternal feed intake to 60% of ad libitum throughout gestation results in a 35% reduction in body weight and a 20–25% reduction in fibre number of fast muscles (Ward and Stickland 1991; Dwyer and Stickland 1992*a*). The reduction in fibre number is known to be due to a reduction in the secondary fibre component of total muscle fibre number (Wigmore and Stickland 1983; Handel and Stickland 1987; Wilson *et al.* 1988; Ward and Stickland 1991). Secondary fibre formation is limited by the availability of secondary myoblasts (Penney *et al.* 1983; Madgwick and Stickland 1988) which suffer impaired proliferation with undernutrition (Madgwick and Stickland

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988). This suggests that the early period of myofibre evelopment, when proliferation of secondary myoblasts ccurs, may be the critical time for the development of ormal numbers of myofibres.

Recent work in the pig has shown that administration f exogenous growth hormone to pregnant sows within ie first third of gestation results in an increase in emitendinosus muscle fibre number in piglets at birth Rehfeldt *et al.* 1992). Muscle fibre number was not ffected by treatment later in gestation. Furthermore, in uman pregnancy, interest has centred on the role of first imester nutrition in preventing low birth weight (Wynn *al.* 1991), and for adequate fetal development of, for xample, the immune system (Chandra 1991). These esults indicate that factors acting during the first third f gestation may be important for establishing optimum etal growth.

The aim of this investigation was to test the hypothesis iat the early period, the first third of gestation, is the ost important time for the development of muscle fibre umbers. This was achieved by assessing muscle fibre umber in the offspring of guinea-pigs undernourished or the first 25 days of gestation, and comparing them ith fetal guinea-pigs undernourished after Day 25, or roughout gestation. A preliminary communication of ome of these results has already appeared (Dwyer *et* . 1993). A second experiment was carried out to xamine whether a shorter early period of nutritional striction would affect fibre number development, and investigate the effect on placental development as a ossible mechanism.

Materials and Methods

This project involved female Dunkin Hartley guinea-pigs weighing tween 700 and 850 g, obtained from a commercial breeder. Guinea-gs were post-partum mated in a harem, where they remained for days. On Day 2 of gestation animals were removed and housed dividually in plastic cages. All animals were fed SG1 pellets rain Harvesters, Wingham, Kent, UK) enriched with vitamin C, d received a vitamin E supplement daily in their drinking water 0 mg L^{-1}; Roche Products Ltd). In the first experiment, pregnant males were randomly assigned to one of four nutritional treatments: ntrols (C)-ad libitum access to feed throughout gestation; TR-pair-d to a weight-matched control at 60% of the ad libitum intake roughout gestation; ER-pair-fed at a 60% ad libitum intake until ay 25 of gestation, then ad libitum to term; LR-ad libitum until ay 25, then 60% ad libitum to term.

The gestation period of the Dunkin–Hartley guinea-pig is 68 days. 25 days' gestation the muscle anlage can be distinguished, but condary myotubes have not started to form and are not seen until ys 30–35 (Ward and Stickland 1991). Dams were allowed to litter d neonates ($n = 52$) were immediately killed by an intra-peritoneal ection of sodium pentobarbitone (Euthesate, 1 mL kg^{-1} body ight). Body weight, and the wet weights of the biceps brachii, eus and liver were determined. In addition, the lengths of the merus and tibia and the crown–rump length were measured. The t biceps brachii and soleus muscles were exposed in each neonate d fixed *in situ* with 2·5% glutaraldehyde. Muscles were then removed and post-fixed in 0·6% osmium tetroxide in 0·4% potassium ferrocyanide. Muscles were dehydrated through graded acetones and embedded in araldite resin (TAAB Laboratories Ltd, Aldermaston, Berks, UK). Resin sections allowed accurate measurements into the muscle to be made so that all sections were taken from the same region in the mid belly of the muscle. 1-μm thick semithin transverse sections were cut and stained with 1% toluidine blue in 1% borax solution. The total number of muscle fibres in each muscle was determined by direct counting of all fibres.

In a second experiment, 8 pregnant guinea-pigs were fed at 60% of ad libitum until Day 15 of gestation and then rehabilitated to an ad libitum regime (VER). The body weight of these dams was recorded at weekly intervals. Five dams were killed at term, by an i.p. injection of Euthesate, and full-term fetuses and placentae were obtained. The remaining 3 pregnant guinea-pigs were killed at Day 45 of gestation, and fetuses and placentae were removed. Placental efficiencies (fetal weight supported per g of placenta, Mellor 1983) were calculated at each age. Fetal parameters were measured as above for neonates and fetal muscles were prepared as described. In addition, cryostat sections (10 μm) of Day-45 muscles were stained to demonstrate myosin ATPase, after Guth and Samaha (1970), to allow the histochemical determination of primary and secondary fibres (as described by Ward and Stickland 1991). The mean ratio of secondary fibres to primary fibres was estimated for each animal. Sagittal sections through the widest part of the placental discs of placentae from Day-45 and term pregnancies were prepared for analysis of structural components as previously described (Dwyer *et al.* 1992). Placentae from VER animals were compared with placentae from control and TR animals at a similar gestational age. Nutritional groups were compared by Newman–Keul ANOVA.

Results

Results from the two experiments outlined above are combined for full-term animals unless otherwise stated. Pregnancies in the VER, ER and TR groups appeared essentially normal and resulted in mean litter sizes of 3·5 pups. The LR group was complicated by a drastic reduction in maternal weight when feed restriction was imposed at Day 25, and by a high level of fetal resorptions such that only 3 of 12 litters were carried to term, yielding 8 neonates. The results obtained from this small group of neonates are included; caution, however, must be exercised in the interpretation of these results. In addition, the viable litters in this group littered on average 6 days earlier than other groups ($P < 0·05$; Newman–Keul ANOVA).

Gross Parameters

Term. The reduction in body and organ weights and lengths is shown in Fig. 1 and Table 1. All restricted groups were significantly smaller than control neonates. In general, the size order was C > ER = VER > TR > LR. LR neonates tended to be 50% smaller than controls, TR were 40% smaller and ER and VER 12% smaller. Lengths were also reduced to the order of 5, 8, 20 and 27% for VER, ER, TR and LR neonates respectively. Owing to the premature littering of the LR group, these neonates were also compared with a group of fetuses of a similar chronological age (approximately 62 days

of gestation) that had been undernourished throughout gestation. Mean fetal body weight was 47·90 g, which did not differ significantly from mean LR neonate weight of 41·35 g ($P = 0·128$, unpaired t-test).

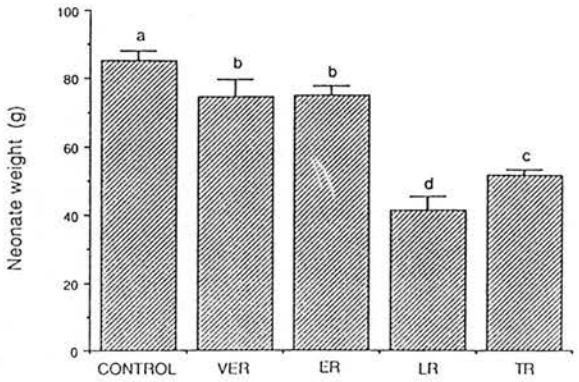


Fig. 1. Mean and standard errors for neonatal body weight for guinea-pig nutritional groups. Significant differences ($P < 0·05$) are indicated by differing letters.

45 days. The weights, and other parameters, of fetuses obtained at 45 days of gestation are shown in Table 2. Surprisingly the VER fetuses at this age were significantly heavier than both similarly aged control (20%) and TR (40%) fetuses ($P < 0·05$). Body lengths were also longer in the VER group by 9·5%, but the length of the long bones and muscle weights were similar in control and VER fetuses.

Muscle Fibre Numbers

The reduction in biceps brachii fibre numbers at term is shown in Fig. 2. Fibre numbers were significantly reduced by 28%, 20% and 25% for ER, TR and LR groups respectively, but fibre number was not affected in the VER group. Soleus fibre numbers were not significantly affected by any of the nutritional treatments (data not shown). At 45 days' gestation VER fetuses had biceps and soleus fibre numbers and secondary-to-primary fibre number ratios similar to those of control animals (Table 3), but biceps fibre number and secondary-to-primary fibre number ratio were reduced in the TR fetuses.

Effects on Maternal Weight

The changes in maternal weight of control, VER and TR dams, as a percentage of their weight at conception, are shown in Fig. 3. During the first third of gestation there was little change in maternal weight in the control group, whereas both restricted groups showed a loss of approximately 10% of their pre-pregnancy body weight. During the last two-thirds of gestation, control and TR dams showed a continuous, approximately linear growth rate (Fig. 3). The VER group, however, gained weight

Table 1. Body lengths and organ weights of control and restricted neonates of guinea-pigs

Values are means, with standard errors in parentheses. Significant differences ($P < 0·05$), determined by Newman–Keul ANOVA, are indicated by differing superscript letters. CR, crown–rump; for treatment codes see Materials and Methods

	Control	VER	ER	LR	TR
Number	27	15	15	8	30
Biceps wt (mg)	56·16 (2·63)^{a}	50·15 (4·34)^{a}	48·60 (1·99)^{a}	24·75 (4·99)^{b}	29·21 (1·89)^{b}
Soleus wt (mg)	24·07 (0·91)^{a}	19·14 (0·94)^{b}	19·60 (1·61)^{b}	11·71 (2·32)^{c}	14·06 (1·00)^{c}
Liver wt (g)	4·48 (0·19)^{a}	4·46 (0·40)^{a}	3·79 (0·10)^{ab}	3·09 (0·53)^{bc}	2·89 (0·19)^{c}
CR length (mm)	110·58 (1·15)^{a}	105·30 (0·26)^{b}	102·0 (1·13)^{b}	80·88 (3·96)^{d}	87·80 (1·33)^{c}
Humerus length (mm)	22·15 (0·62)^{a}	19·82 (0·36)^{b}	18·87 (0·66)^{b}	14·75 (0·82)^{c}	18·16 (0·49)^{b}
Tibia length (mm)	27·53 (0·30)^{a}	24·14 (0·38)^{b}	24·73 (0·68)^{b}	19·75 (0·84)^{c}	23·77 (0·37)^{b}

Table 2. Body lengths and organ weights of control and restricted guinea-pig fetuses at 45 days of gestation

Mean (s.e.). Significant differences ($P < 0·05$), determined by Newman–Keul ANOVA, are indicated by differing superscript letters. CR, crown–rump; for treatment codes see Materials and Methods

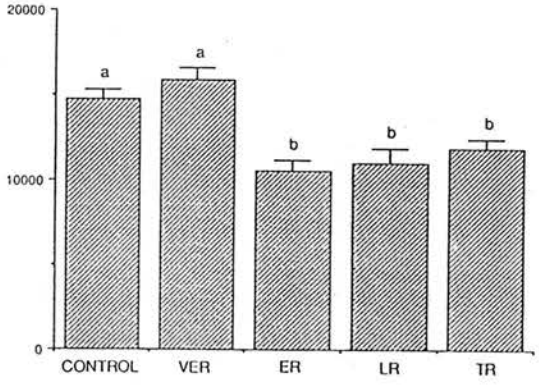
	Control	VER	TR
Number	11	12	16
Body wt (g)	20·53 (1·01)^{b}	24·78 (0·82)^{a}	16·58 (0·33)^{c}
Placenta wt (g)	3·68 (0·24)^{a}	3·53 (0·08)^{a}	3·69 (0·22)^{a}
Fetal : Placental wt	5·72 (0·32)^{b}	7·00 (0·19)^{a}	4·68 (0·23)^{c}
Biceps wt (mg)	16·55 (1·31)^{ab}	18·93 (0·99)^{a}	13·88 (1·56)^{b}
Soleus wt (mg)	9·55 (0·80)^{a}	9·28 (1·08)^{a}	4·97 (1·00)^{b}
Liver wt (g)	1·46 (0·07)^{b}	2·05 (0·06)^{a}	1·15 (0·04)^{c}
CR length (mm)	66·09 (1·66)^{b}	72·33 (1·10)^{a}	60·88 (0·73)^{c}
Tibia length (mm)	15·84 (1·32)^{a}	15·48 (0·25)^{a}	13·31 (0·55)^{a}
Humerus length (mm)	14·16 (1·11)^{a}	13·77 (0·14)^{a}	11·16 (0·39)^{b}

Table 3. Muscle fibre number and secondary-to-primary fibre number ratio (S:P) data of 45-day-old guinea-pig fetuses from control, VER and TR dams

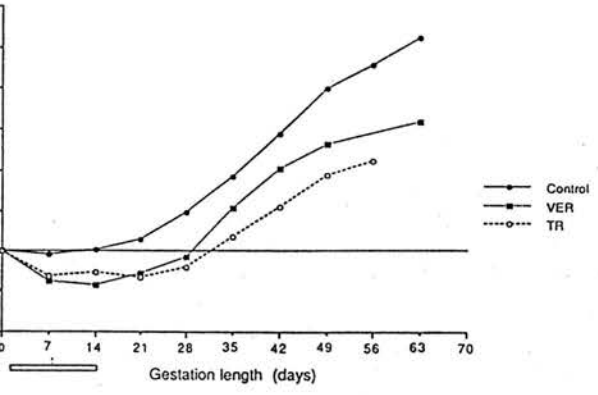
Mean (s.e.). Significant differences ($P < 0.05$) are indicated by differing superscript letters. For treatment codes see Materials and Methods

Muscle	Control	VER	TR
Biceps fibre number	11385 (848)[a]	12256 (728)[a]	8362 (566)[b]
Biceps S:P ratio	8·21 (0·42)[a]	7·57 (0·33)[a]	6·14 (0·14)[b]
Soleus fibre number	2221 (862)[a]	3313 (153)[a]	2339 (720)[a]
Soleus S:P ratio	1·67 (0·15)[a]	1·61 (0·12)[a]	1·63 (0·06)[a]

dly in the second third, at a rate comparable to rol dams, but showed a significantly slower gain over last third of gestation (Table 4). By term, control s showed a mean net weight gain of 16·23% (1·82 y^{-1}) when the conceptus weight was removed. VER TR dams showed a mean net weight loss of 6·39% 9 g day^{-1}) and 13·68% (1·62 g day^{-1}) respectively.



2. Mean and standard errors for neonate biceps brachii fibre er for guinea-pig nutritional groups. Significant differences 0·05) are indicated by differing letters.



. Mean changes in maternal weight for control, TR and VER a-pig dams throughout gestation expressed as a percentage of pre-pregnancy body weight. Bar represents the duration of tion in the VER group.

ts on Placental Components

ne effect of undernutrition on placental component ne is shown in Fig. 4 and on weight in Fig. 5. At 45 days of gestation, 30 days after the period of restriction in the VER group, placenta weights were similar in all three groups (Fig. 5). However, in the VER group the placenta was composed of a larger volume of peripheral labyrinth (PL) tissue (Fig. 4) than the other groups (14%, $P < 0.05$). This was largely at the expense of the interlobium component which was 40% smaller in VER placentae compared with control and TR placentae. The volume of the central labyrinth (CL) was also increased to 4% of the placental volume, compared with 2% in other groups, though large variation between individuals meant this did not reach significance ($P = 0.18$). Thus, at 45 days' gestation, the interlobium weight was reduced in VER placentae, and peripheral and central labyrinth weights were similar to controls and heavier than TR placentae (Fig. 5).

Table 4. Maternal average daily weight gains throughout gestation for control, VER and TR guinea-pigs

Mean values in g day^{-1}. For treatment codes see Materials and Methods

Stage of gestation	Control	VER	TR
Days 0–25	0·85	−2·19	−2·20
Days 25–45	9·03	9·77	6·28
Day 45–term	10·91	4·44	6·36

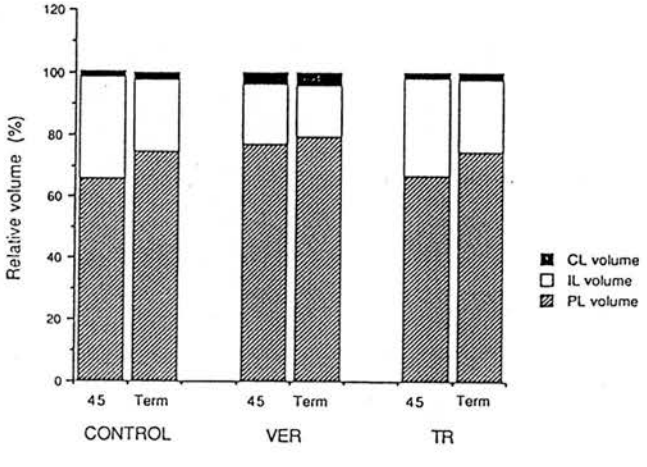


Fig. 4. Relative volumes of placental components at 45 days' gestation and at term for control, TR and VER guinea-pig pregnancies. CL, central labyrinth; PL, peripheral labyrinth; IL, interlobium. See text for significant differences.

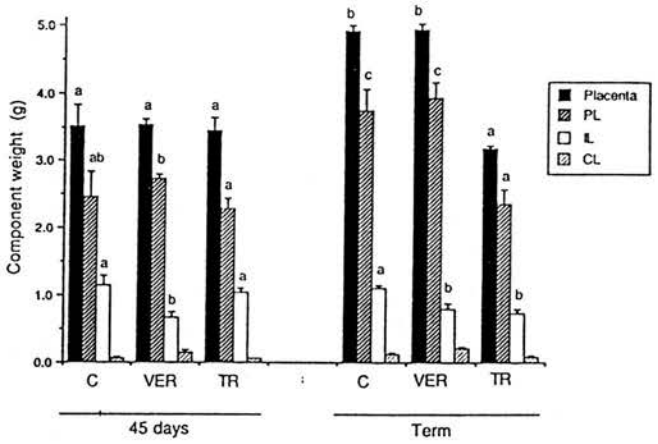


Fig. 5. Mean (and standard error bars) placental component weights at 45 days' gestation and at term for control, TR and VER guinea-pig placentae. Significant differences ($P < 0{\cdot}05$) between groups and ages for each component are indicated by differing letters.

At term, placental weight had increased by approximately 30% in the VER and control groups but was unchanged in TR placentae. PL weight was also increased in control and VER by an increase in proportional volume and weight of tissue in control, but by an increase in weight alone in VER (Figs 4 and 5). Both control and TR groups showed an increase in PL proportional volume (10%) at the expense of the interlobium from Day 45, but the VER group was unchanged.

Placental efficiencies were elevated in the VER group at Day 45 (Table 2) compared with both control and TR groups. The TR placentae were significantly less efficient than both control and VER placentae. At term, efficiencies were similar in the VER and restricted TR placentae ($16{\cdot}43 \pm 0{\cdot}62$ and $17{\cdot}48 \pm 0{\cdot}45$, respectively), and significantly lower than in the control group ($19{\cdot}71 \pm 0{\cdot}81$; $P < 0{\cdot}05$).

Discussion

In this study the hypothesis that the early period of gestation, the first 25 days in the guinea-pig, is critical for the development of fetal muscle fibre numbers was initially tested by investigating the effects of a 40% reduction in maternal feed intake imposed before or after 25 days of gestation. All restricted groups were found to be significantly smaller than controls fed ad libitum (Fig. 1 and Table 1) and had fewer muscle fibres in the biceps brachii (Fig. 2). If the period of undernutrition was imposed only for the first 15 days of gestation (the VER group) body weight was reduced at term by a similar degree to the ER group (Fig. 1), but biceps fibre number was unaffected (Fig. 2). The soleus muscle was found to be unaffected by any of the nutritional treatments used. This is probably due to the low ratio of secondary fibres to primary fibres of this slow-twitch muscle. Slow muscles are never as affected by undernutrition as fast muscles (Bedi *et al.* 1982; Ward and Stickland 1991; Dwyer and Stickland 1992*a*), since slow muscles contain a greater relative proportion of primary fibres (Kelly and Rubenstein 1986), and primary fibres are unaffected by undernutrition (Handel and Stickland 1987). Restriction of maternal feed intake for the first 25 days alone (ER) was sufficient to reduce biceps fibre numbers by a similar degree to restriction throughout gestation. There was, therefore, no evidence that nutritional rehabilitation, after Day 25, had an effect on fibre numbers in the guinea-pig fetus. Some recovery of fibre number has been reported on refeeding before the end of fibre hyperplasia in the rat (Wilson *et al.* 1988). This does not appear to be the case in the guinea-pig since biceps fibre numbers were preserved only when rehabilitation occurred well before the onset of myofibre hyperplasia, from 15 days' gestation onwards (VER). It is assumed that the deficit in fibre numbers was in the secondary fibre component, since undernutrition throughout gestation has been shown to cause a decrease in secondary fibres only (Ward and Stickland 1991).

In the guinea-pig the maximum rate of secondary myofibre formation in the biceps occurs between Days 35 and 45 of gestation (Ward and Stickland 1991). The rate of fibre formation, and the ultimate number of fibres formed, is dependent on the availability of myoblasts (Penney *et al.* 1983). In double-muscled cattle, myoblasts undergo a prolonged proliferative phase (Quinn *et al.* 1990), which results in a greater number of terminally differentiated myoblasts and, hence, a greater number of muscle fibres than in control cattle (Shahin and Berg 1985). In addition, maternal undernutrition throughout gestation in the guinea-pig is known to reduce myoblast proliferation (Madgwick and Stickland 1988) leading to a reduction in the number of muscle fibres formed in the fetus (Ward and Stickland 1991; Dwyer and Stickland 1992*a*). In the present study, nutritional insufficiency before fibre formation (ER) caused a reduction in secondary fibre numbers (Fig. 2). Undernutrition, therefore, may have affected secondary myoblast proliferation thereby reducing the potential number of secondary fibres which could form. However, a recent study (Madgwick and Stickland 1988) suggested that there was no deficit in myonuclear numbers before Day 40 of gestation in the undernourished guinea-pig fetus. Therefore, a reduction in the number of secondary myoblasts seems unlikely to have occurred at Day 25 of gestation in the ER group.

An alternative hypothesis is that early undernutrition had a detrimental effect on the establishment and growth of the feto-placental unit. This is supported by the significantly reduced size of the ER and VER neonates compared with the controls. The most rapid phase of fetal growth is in the last half of gestation (Block *et*

1988; Ward and Stickland 1991) and well after the od of nutritional restriction in the ER group, yet ER nates were unable to achieve growth rates similar to e of controls. This may have been due to a reduced ental size which may have led to impaired fetal ition throughout gestation. Maternal undernutrition es a reduction in placental mass, DNA content and nange surface area in rats and guinea-pigs (Rosso 1980; yer *et al.* 1992). This results in reduced placental sfer to the fetus (Saintonge and Rosso 1981). In revious study, maternal feed restriction throughout ation caused impaired placental efficiency in the ea-pig by Day 25 of gestation (Dwyer *et al.* 1992), mpanied by a reduced exchange surface area. In the R group, changes in placental structure were seen at 45 (Figs 4 and 5): the relative volumes of the PL CL had increased and of the IL had decreased. Fig. 1 onstrates the similarity of the proportional changes in ental component volumes between Day 45 and term ne control and TR groups, such that any differences mponent weights at term were due to the increase in weight of the control placenta (Fig. 5). The changes in the VER group, however, suggest that some tation of placental function occurs with nutritional iction and subsequent rehabilitation. The placenta eases in size until approximately Day 50 in the ea-pig (Dwyer *et al.* 1992). Cell numbers, however, h a maximum and plateau before placental weight in mouse (Iguchi *et al.* 1993) and rat placenta (Winick Noble 1966). Changes in placental morphology are plete by Day 35 in the guinea-pig (Kaufmann and doff 1977), which may coincide with the end of division. This suggests that nutritional restriction ends on Day 15 of gestation may permit adequate ental rehabilitation and adaptation such that no loss myofibre number occurs (VER). However, in the ER p the restoration of an ad libitum diet may be too to allow adequate placental compensation. Hence, may be a critical period for the development of placenta.

he effect of maternal undernutrition on the development ne placenta also explains why there is no effect of tional rehabilitation on muscle fibre numbers in the ea-pig, unlike the rat (Wilson *et al.* 1988). In the ea-pig, secondary fibre hyperplasia is completed *in* such that the fetus is permanently stunted by a ced placental size, despite maternal rehabilitation. ne rat, secondary fibre proliferation continues into postnatal life; therefore, early placental stunting not have a permanent effect on muscle fibre number habilitation occurs after birth.

espite placental adaptations which allow normal, or accelerated, fetal growth at mid term (Table 2), ull term the VER group were significantly smaller than controls, and their placentae were significantly less efficient at supporting fetal growth. A possible explanation may lie in the inability of the early-restricted dams to deposit fat in the early stages of gestation (Fig. 3). Pregnant guinea-pigs rapidly increase their feed intake within 5 days of conception, reaching a plateau at Day 45 of gestation (unpublished observation). Unfortunately, feed intake in the VER group after rehabilitation was not measured, but it is likely that there was some compensatory increase in intake, at least in the early stages of refeeding. The VER group dams showed a weight loss during the first third of gestation similar to that in the TR group (Fig. 3), and a reduced weight gain over the last third (Table 4). In the guinea-pig, total litter weight may be as much as 50% of the pre-pregnancy body weight of the dam (Leitch *et al.* 1959) and it is unlikely that the dam can support this nutritional burden without drawing on her own body resources. The VER and ER groups, with inadequate early gestational fat deposition, were probably unable to support rapid fetal growth in late gestation, regardless of their feed intake, resulting in a reduction of fetal body weight.

Restriction of maternal intake from Day 25 onwards caused a large number of fetal resorptions, and severe growth retardation in those animals which were carried to term. Conclusions drawn from these data are necessarily limited, because of the drastic effects the imposition of undernutrition had on the maintenance of pregnancy. The very small size of the LR neonates was mainly due to the premature birth and younger chronological age of these animals. However, comparison of these neonates with undernourished fetuses of a similar age suggests that other factors may also be involved. A good level of maternal nutrition in the first third of gestation leads to good placental growth and development (Dwyer *et al.* 1992), which cannot then be supported on a lower nutritional plane without loss of nutrient supply to the fetus. It is possible that the LR fetuses had an exceptionally low feto–placental weight ratio, which may have compounded the restriction felt by the LR animals. This may have also induced the high degree of fetal resorptions seen in this group. An additional factor may have been maternal and fetal stress, caused by undernutrition following previous good nutrition, and this situation merits further investigation.

Those LR animals that did reach term had a reduced biceps brachii fibre number when compared with control animals (Table 2). Restriction during the period of secondary fibre hyperplasia (Ward and Stickland 1991), therefore, also appears to cause a reduction in the number of myotubes that are formed. This may have been mediated by actions of the insulin like growth factors (IGF–I and–II). Receptors for the IGFs have been seen as early as the morula stage of the mouse embryo (Heyner

et al. 1990), and IGF–I and–II are known to stimulate proliferation of L6 myoblasts *in vitro* (Ewton *et al.* 1987). Maternal fasting, intra-uterine growth retardation by uterine artery ligation or maternal undernutrition of rats and guinea-pigs cause a reduction in fetal serum IGF–I (Davenport *et al.* 1990; Jones *et al.* 1990; Bernstein *et al.* 1991; Dwyer and Stickland 1992*b*). It is possible, therefore, that maternal undernutrition, after Day 25 of gestation, caused a reduction in fetal IGF–I, which in turn led to a reduced proliferation of secondary myoblasts between Days 35 and 50 when secondary myotubes are formed (Ward and Stickland 1991). The same mechanism for a reduction in myofibre number probably operates in the ER group, but the causes of undernutrition, during the period of fibre formation, are different.

In conclusion, maternal feed restriction for the first 25 days of gestation alone caused a reduction in neonate myofibre number equal to that seen in animals restricted throughout gestation. However, restriction for only the first 15 days of gestation did not prevent the development of the normal number of myofibres. Restriction from Day 25 onwards also caused a reduction in myofibre number, and a severe retardation of fetal growth. Undernutrition before Day 25 probably exerts its main effects on the development of the placenta and, therefore, indirectly on secondary fibre proliferation owing to the consequent undernutrition of the fetus later in gestation, caused by impaired placental nutrient transfer. Undernutrition imposed after Day 25 appears to be at near-fatal severity, owing to the well developed placenta formed earlier in gestation when *ad libitum* feed access was permitted. The effect on fibre development in the LR group is likely to be a direct consequence of maternal undernutrition occuring at the time of fibre production.

Acknowledgments

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References

Bedi, K. S., Birzgalis, A. R., Mahon, M., Smart, J. L., and Wareham, A. C. (1982). Early life undernutrition in rats. I. Quantitative histology of skeletal muscles from underfed young and refed adult animals. *Br. J. Nutr.* **47**, 417–30.

Bernstein, I. M., DeSouza, M. M., and Copeland, K. C. (1991). Insulin-like growth factor I in substrate-deprived, growth-retarded fetal rats. *Pediatr. Res.* **30**, 154–7.

Block, S. M., Johnson, R. L., Sparks, J. W., and Battaglia, F. C. (1988). Uterine metabolism of the pregnant guinea-pig as a function of gestational age. *Pediatr. Res.* **23**, 45–9.

Chandra, R. K. (1991). Interactions between early nutrition and the immune system. *Ciba Found. Symp.* **156**, 77–92.

Davenport, M. L., D'Ercole, A. J., and Underwood, L. E. (1990). Effects of maternal fasting on fetal growth, serum insulin-like growth factors and tissue insulin-like growth factor mRNAs. *Endocrinology* **126**, 2062–7.

Dwyer, C. M., Madgwick, A. J. A., Crook, A. R., and Stickland, N. C. (1992). The effect of maternal undernutrition on the growth and development of the guinea pig placenta. *J. Dev. Physiol.* **18**, 295–302.

Dwyer, C. M., Madgwick, A. J. A., Ward, S. S., and Stickland, N. C. (1993). The effect of maternal undernutrition, imposed before or after the first trimester, on muscle fibre number development in the guinea pig fetus. *J. Anat.* **183**, 200 [Abstr.].

Dwyer, C. M., and Stickland, N. C. (1992*a*). Does the anatomical location of a muscle affect the influence of undernutrition on muscle fibre number? *J. Anat.* **181**, 373–6.

Dwyer, C. M., and Stickland, N. C. (1992*b*). The effect of maternal undernutrition on maternal and fetal serum insulin-like growth factors, thyroid hormones and cortisol in the guinea pig. *J. Dev. Physiol.* **18**, 303–13.

Ewton, D. Z., Falen, S. L., and Florini, J. R. (1987). The type II Insulin-like growth factor receptor has a low affinity for IGF–I analogues: pleiotypic actions of the IGFs on myoblasts are apparently mediated by the type I receptor. *Endocrinology* **120**, 115–23.

Guth, L., and Samaha, F. J. (1970). Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Exp. Neurol.* **28**, 365–7.

Handel, S. E., and Stickland, N. C. (1987). Muscle cellularity and birthweight. *Anim. Prod.* **44**, 311–17.

Heyner, S., Farber, M., and Rosenblum, I. Y. (1990). The insulin family of peptides in early mammalian development. *Curr. Top. Dev. Biol.* **24**, 137–59.

Iguchi, T., Tani, N., Sato, T., Fukatsu, N., and Ohta, Y. (1993). Developmental changes in mouse placental cells from several stages of pregnancy *in vivo* and *in vitro*. *Biol. Reprod.* **48**, 188–96.

Jones, C. T., Lafeber, H. N., Rolph, T. P., and Parer, M. (1990). Studies on the growth of the fetal guinea- pig. The effects of nutritional manipulation on prenatal growth and plasma somatomedin activity and IGF concentrations. *J. Dev. Physiol.* **13**, 189–97.

Kaufmann, P., and Davidoff, M. (1977). The guinea pig placenta. *Ergeb. Anat. Entwicklungsgesch.* **53**, 5–91.

Kelly, A. M., and Rubenstein, N. A. (1986). Muscle histogenesis and muscle diversity. In 'Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology'. (Eds C. Emerson, D. Fischman, B. Nadal–Ginard and M. A. Q. Siddiqui.) Vol. 29, pp. 77–84. (Alan R. Liss: New York.)

Leitch, I., Hytten, F. E., and Billewicz, W. Z. (1959). The maternal and neonatal weights of some mammalia. *Proc. Zool. Soc. London* **133**, 11–28.

Madgwick, A. J. A., and Stickland, N. C. (1988). The effect of undernutrition on nuclear populations in fast and slow twitch muscles during myogenesis in the guinea pig. *J. Anat.* **161**, 226–7 [Abstr.].

Mellor, D. J. (1983). Nutritional and placental determinants of fetal growth rate in the sheep and consequences for the newborn lamb. *Br. J. Nutr.* **139**, 307–24.

Penney, R. K., Prentis, P. F., Marshall, P. A., and Goldspink, G. (1983). Differentiation of muscle and the determination of ultimate tissue size. *Cell Tissue Res.* **228**, 375–88.

Quinn, L. S., Ong, L. D., and Roeder, R. A. (1990). Paracrine control of myoblast proliferation and differentiation by fibroblasts. *Dev. Biol.* **140**, 8–19.

Rehfeldt, C., Fiedler, I., Weitkard, R., Spitschak, K., and Ender, K. (1992). Effects of pST administration to pregnant sows on developmental stage and *semitendinosus* muscle cellularity of the newborn piglets. 38th International Congress on Meat Science and Technology, Clermont–Ferrand, France.

Rosso, P. (1980). Placental growth, development and function in relation to maternal nutrition. *Fed. Proc.* **39**, 250–4.

Saintonge, J., and Rosso, P. (1981). Placental blood flow and transfer of nutrient analogs in large, average and small guinea-pig littermates. *Pediatr. Res.* **15**, 152–6.

Shahin, K. A., and Berg, R. T. (1985). Growth patterns of muscle, fat and bone, and carcass composition of double-muscled and normal cattle. *Can. J. Anim. Sci.* **65**, 279–94.

Ward, S. S., and Stickland, N. C. (1991). Why are fast and slow muscles differentially affected during prenatal undernutrition? *Muscle and Nerve* **14**, 259–67.

Wigmore, P. M. C., and Stickland, N. C. (1983). Muscle development in large and small pig fetuses. *J. Anat.* **137**, 235–45.

Wilson, S. J., Ross, J. J., and Harris, A. J. (1988). A critical period for the formation of secondary myotubes defined by prenatal undernourishment in rats. *Development* **102**, 815–21.

Winick, M., and Noble, A. (1966). Quantitative changes in ribonucleic acids and protein during normal growth of rat placenta. *Nature (Lond.)* **212**, 34–5.

Wynn, A. H., Crawford, M. A., Doyle, W., and Wynn, S. W. (1991). Nutrition of women in anticipation of pregnancy. *Nutr. Health* **7**, 69–88.

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1–3 *April* 1981. J. Anat., (1981), **133**, 1, p. 132]

49. Prenatal muscle development in the pig: a comparison of the largest and smallest litter mates.
By P. M. C. WIGMORE and N. C. STICKLAND. *Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh*

The semitendinosus muscle was removed from both sides of the largest and smallest fetus in litters ranging from 38 days gestation to full term. One muscle was used to obtain frozen transverse sections for histological examination and the other muscle was used for biochemical analyses.

Measurements on the frozen sections showed that cell diameter in the small fetuses was smaller than that in the large, but this difference was largely correlated with the considerable fetal weight differences. The biochemical analyses showed that protein, RNA and DNA contents were also weight-related rather than age-related parameters.

Histological examination of the muscle sections revealed the expected biphasic development of muscle fibres, with primary myotubes being present at 38 days, and secondary fibres forming between 50 and 55 days gestation. In large and small fetuses the myotubes showed an increase in diameter of 144 % and 90 % respectively from the 38 days stage, before decreasing. As well as the difference in the amount of increase, it was noted that the maximum myotube diameter was attained significantly later in the small fetuses. Although myotubes from the large fetuses had the greater myofibrillar content they had also a significantly greater proportion of intracellular space (or non-myofibrillar content). The effect of these factors on myotube diameter may be important since secondary myofibres are known to form on the surfaces of myotubes. The reduced myotube surface area in the small fetuses may therefore be a factor contributing to their reduced fibre number at full term.

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18. A scanning electron microscopic study of prenatal muscle development *in vivo*. By N. C. STICKLAND. *Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh* (Fig. 8).

Early stages of myoblast fusion have been studied in tissue culture preparations and some of these studies have employed scanning electron microscopy. Structural aspects of muscle development *in vivo*, however, have been studied almost exclusively using sectioning techniques. Theories of muscle development, based on these latter studies, include deductions about the longitudinal arrangement of cells and fibres in developing muscle. Scanning electron microscopy of muscle tissue *in vivo* provides a method whereby our knowledge of the three dimensional arrangement of developing muscle may be extended.

Mouse fetuses (C57 strain) were obtained at daily intervals from 12 days gestation to almost full term (18 days). Whole forelimbs were dissected off the fetuses and fixed in 3 % glutaraldehyde. In the older fetuses (> 15 days) m. biceps brachii was then removed entirely whereas in the younger fetuses the muscle was left *in situ*. The muscles were pulled apart to expose the developing muscle fibres and then processed and critical point dried. Freeze-drying techniques were also

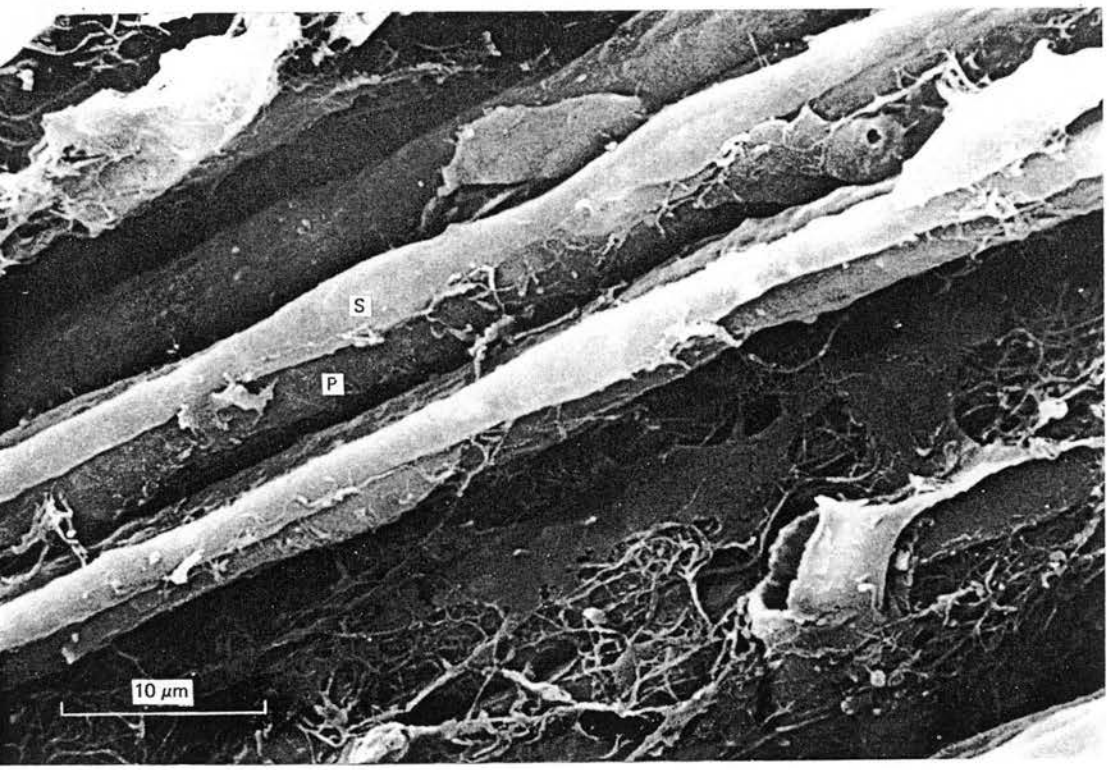

Fig. 8

attempted but did not produce such good results. The muscles were viewed on an ISI-60 scanning electron microscope.

At 12 days muscle tissue is difficult to distinguish whereas at 13 days long fibres are evident. Spindle shaped cells (myoblasts) can be seen in the crevices between adjacent fibres. At 16 and 17 days a population of secondary smaller fibres (Fig. 8, S) can be seen forming on the surfaces of the primary fibres (Fig. 8, P). At 18 days the fibres are more compact, with some fibres appearing more intimately associated with one another, possibly as the basal lamina enveloping adjacent primary and secondary fibres becomes more pronounced.

J. Anat. 155, 240

D. 12. Backscattered electron imaging of nuclei in prenatal muscle development. By S. C. BROWN and N. C. STICKLAND. *Department of Anatomy, The Royal Veterinary College, London* (Fig. 3).

Prenatal development of m. biceps brachii in the mouse was investigated from 15 days gestation up until 19 days (full term), using both secondary and backscattered electron imaging. Secondary electrons provide information on the surface of the specimen whereas the backscatter mode used in conjunction with a metal stain detects density differences from just below the surface (Fig. 3). A silver stain specific for nuclei (De Nee *et al. Scanning Electron Microscopy* 1974 (Pt 1), Proceedings of the Seventh Annual Scanning Electron Microscope Symposium, April 1974) was used to visualise nuclei within the developing myotubes. This technique, together with transmission electron microscope studies, has enabled nuclear distribution to be evaluated in developing primary and secondary myotubes.

At 15 days gestation irregularly spaced mononucleated cells align on the surfaces of the primary myotubes and subsequently fuse to form the secondary myotubes by 16 days. After fusion the nuclei in the secondaries become evenly distributed but with greater spacing than that seen in the primaries at this stage. With subsequent increase in size of the secondary myofibres mononucleated cells fuse along their length so that at birth all fibres are of a similar size and exhibit similar nuclear spacing. Differences in density of nuclei at this stage (Fig. 3) may be related to the different types of nuclei present.

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J. Anat. 155, 240

D. 11. The effect of undernutrition on fibre number in myogenesis. By S. S. WARD and N. C. STICKLAND. *Department of Anatomy, The Royal Veterinary College, London*

Myogenesis is a biphasic process in which myoblasts fuse to form an initial population of myotubes (the primary fibres) upon which further populations develop (secondary fibres). It has been suggested that malnutrition operates to reduce total fibre number in a muscle during this prenatal, hyperplastic period by limiting the number of secondary fibres formed. Until now this has not been proved in a nutritionally controlled experiment.

In the present study, groups of pregnant guinea-pigs were pair-fed on a reduced diet of 60% of *ad lib.* through gestation and killed at 5 day intervals from 30 days gestation to term (68 days average). The biceps brachii muscle of each fetus was prepared for analysis of fibre number, size and secondary to primary fibre ratio using electron microscopy and enzyme histochemistry.

Fetuses of reduced diet mothers were progressively lighter in weight than their *ad lib.* contemporaries with advancing gestational age. This was reflected in the effect on the muscle which becomes more acute towards parturition. Initial results support previous findings on the mode of decreased fibre number. There is a reduced secondary to primary fibre ratio in the nutritionally disadvantaged muscle at every age examined, together with a reduction in primary fibre size of about 27%.

This reduction helps to explain the decrease in fibre number at birth which is in the order of 30% in the biceps brachii muscle of the nutritionally deficient animal.

This work was supported by the Agriculture and Food Research Council.

SYMPOSIUM: THE GROWTH AND DEVELOPMENT OF SKELETAL MUSCLE

1. Prenatal muscle development and its effect on postnatal growth. By N. C. STICKLAND. *Department of Veterinary Basic Sciences, the Royal Veterinary College, University of London*

The biphasic theory of muscle development is that myoblasts line up and fuse to form an initial population of large primary myofibres. A larger population of small secondary myofibres then forms on their surfaces. In most muscles of mixed fibre types the primary myofibres take on slow fibre characteristics and the secondaries take on fast characteristics (although some secondaries may become slow either prenatally or postnatally).

The effects of genetic and nutritional factors on the above process of myogenesis have been investigated in several species. The genetic control of muscle size has been studied in large and small strains of animals including mice and pigs. The major genetic influence on muscle size appears to be during primary myofibre formation. This is in contrast to the nutritional situation where low levels of maternal nutrition cause a marked reduction in the number of secondary myofibres associated with each primary. The number of primary myofibres is not affected except in very severe cases of runting in the pig. The reduction in secondary myofibre formation is associated with several factors including a reduction in primary myofibre surface area and in nuclear proliferation.

There are several consequences of the effects in prenatal muscle development mentioned above on postnatal muscle growth. An alteration in the ratio of secondary to primary myofibres formed during development has an effect on muscle fibre type ratios in postnatal growth. Work on pigs has shown that animals which develop fewer muscle fibres by the time of birth remain disadvantaged throughout postnatal growth. Small pigs at birth which exhibit catch-up growth contain relatively high numbers of muscle fibres in their muscles. This situation is possible when there are low nutritional levels in only the later part of gestation; this would affect the size but not the number of muscle fibres.

8. The effect of undernutrition on the myogenesis of fast and slow twitch muscles of the guinea-pig.

By S. S. WARD and N. C. STICKLAND. *Department of Veterinary Basic Sciences, Royal Veterinary College, University of London*

Myogenesis is a biphasic process in which secondary fibres form on primary fibres. It has been suggested that undernutrition operates during prenatal muscle development to reduce fibre number by limiting the number of secondary fibres formed. Muscles in which presumptive slow twitch fibres predominate, appear to be exempted from fibre number reduction. The mechanism which spares slow twitch muscles had not yet been investigated in a nutritionally controlled experiment.

In the present study, pregnant guinea-pigs were pair-fed on a reduced diet of 60% of *ad lib.* throughout gestation and killed at 5 day intervals from 30 days' gestation to term (average 68 days). The biceps brachii and soleus muscles (a fast and slow twitch muscle respectively), of each fetus were prepared for analysis of fibre number, size and secondary to primary fibre ratio using electron microscopy and enzyme histochemistry.

The results show clear differences between the muscle types. Biceps brachii muscle fibre number was reduced by approximately 27% at birth, while there was no significant reduction in soleus fibre number. From 40 to 45 days of gestation the secondary-to-primary fibre ratio is lower in the undernourished than in the control animals in both muscle types. Since there was no difference in primary fibre number from the control this result was indicative of a slower rate of secondary fibre formation in the undernourished muscles. Calculations reveal that whilst the rate of fibre formation was reduced, secondary fibres continue to form over a longer period of time in the nutritionally stressed group. This limited the severity of fibre number reduction in the biceps brachii muscle and prevented it in the soleus muscle. Delayed maturation of the undernourished muscles was further illustrated by immature histochemical fibre staining and continuing presence of the central myofibrillar free area (which is a feature of the hyperplastic phase).

Relative sparing of slow twitch muscle from fibre number reductions in response to undernutrition during development is a consequence of the low ratio of secondary to primary fibres which exists in these muscles. Since secondary fibres form a low proportion of the total number, the full complement of fibres can be achieved in the period of delayed secondary fibre formation. This is not possible in the fast twitch biceps brachii muscle where secondary fibres contribute more than 80% of the total number.

This work was supported by the Agricultural and Food Research Council.

J. Anat. 161, 226-227

9. The effect of undernutrition on nuclear populations in fast and slow twitch muscles during myogenesis in the guinea-pig. By A. J. A. MADGWICK and N. C. STICKLAND. *Department of Veterinary Basic Sciences, Royal Veterinary College, University of London*

Myogenesis is a biphasic process where an initial population of myoblasts fuse to form primary fibres upon which secondary fibres develop by further myoblast fusion. A previous study has shown that prenatal undernutrition results in reduced fibre numbers at term by reducing the number of secondary fibres in the biceps; no such reduction in fibre number is seen in the soleus. It is not clear to what extent these results can be explained by the effect of nutrition on nuclear proliferation in these two muscles.

This study investigated the effects of a reduced diet of 60 % *ad lib.* throughout gestation on pair-fed pregnant guinea-pigs. Pairs of animals were killed at 5 day intervals from 35 days' gestation to term (mean 68 days). The biceps brachii (fast) and soleus (slow twitch) muscles were removed from each fetus and processed for light and electron microscopy so that nuclear compartments in the developing muscles could be quantified.

An initial investigation into the partitioning of nuclei into those within fibres, mononuclear cells (predominantly myoblasts), fibroblasts and endothelial cells suggested that each compartment was equally affected by undernutrition. It is therefore reasonable to assume that an estimate of the total number of nuclei is an indicator of the behaviour of nuclei associated with fibre formation.

During the period of primary fibre formation in the biceps, there was no significant difference in total nuclear number. During the period of secondary fibre formation, however, the nuclear population was reduced by 25 % at 45 days of gestation and by 10 % at term in the undernourished animals. This difference at the two ages can be attributed to the addition of nuclei persisting at a

lower rate, for a longer period of time in the diet restricted animals. This is reflected in a slower rate of secondary fibre formation resulting in a 27% reduction in fibre number at term (Ward *et al. J. Anat.* 1988). Further analysis shows that there is no significant difference between the ratio of fibre number:nuclear numbers in *ad lib.* and restricted animals. These results suggest that the availability of myoblasts to form secondary fibres is one of the limiting factors during myogenesis in the biceps.

Events in the biceps contrast markedly with those in the soleus in which there is no significant difference between nuclear numbers and fibre numbers at term. This effect may be related to the lower rate of nuclear proliferation in the soleus of a well fed animal such that a similar proliferative rate can be maintained at lower nutritional planes.

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The effect of maternal undernutrition on the development of placental components in the guinea pig. By C. M. DWYER, A. J. A. MADGWICK, A. R. CROOK and N. C. STICKLAND. *Department of Veterinary Basic Sciences, the Royal Veterinary College, University of London*

Fetal growth is known to be correlated with the increase in size of the placenta and the exchange surface area. Reduction in the growth of the materno-fetal exchange surface area may be a mechanism by which the effects of maternal undernutrition on fetal growth are mediated. In the compact placenta of the guinea pig the exchange surface area is the peripheral labyrinth. The effect of a 40% reduction in maternal feed intake on the growth of the peripheral labyrinth was investigated between gestational days 25 and 65. Sagittal sections of placentae were stained to demonstrate alkaline phosphatase which allowed measurements to be made of the relative volumes of each placental component.

Fetal and placental weights were significantly reduced in the last trimester by 32% and 38% respectively ($P < 0.01$). Placental efficiency in early gestation was significantly impaired in restricted animals but equivalent to ad lib-fed controls by the last trimester. The volume of peripheral labyrinth increased as a proportion of the total placental volume with gestational age. Restricted placentae tended to be composed of a smaller volume of peripheral labyrinth in early gestation. It is suggested that maternal undernutrition results in an impaired or delayed expansion of the peripheral labyrinth in early gestation, resulting in a reduced placental efficiency. By the last trimester the weight of the peripheral labyrinth of restricted animals was reduced by 33% ($P < 0.05$). The weight of the peripheral labyrinth was significantly correlated with fetal weight during the last trimester, suggesting that fetal weight is limited by the size of the peripheral labyrinth in the later stages of gestation.

The effect of maternal undernutrition, imposed before or after the first trimester, on muscle fibre number development in the guinea pig fetus. By C. M. DWYER, A. J. A. MADGWICK, S. S. WARD and N. C. STICKLAND. *Department of Veterinary Basic Sciences, the Royal Veterinary College, University of London*

Muscle fibre hyperplasia occurs early in development in the guinea pig and is complete by d 50 of gestation. A 40% reduction in maternal intake throughout gestation results in a 35% reduction in body weight at term, and a 20–25% reduction in muscle fibre number in fast muscles (Ward & Stickland, *Muscle Nerve* **14**, 1991). The present investigation was designed to test the hypothesis that the first trimester is the most critical period for fibre number determination when undernutrition exerts an influence.

Pregnant dams were assigned to one of the following nutritional treatments on d 1 of gestation: C, ad lib. throughout gestation; TR, 60% of ad lib. throughout; ER, 60% to d 25 when ad lib.; LR, ad lib to d 25 then 60%. Neonates ($n = 52$) were killed, by an overdose injection of sodium pentobarbitone, and body and muscle wet weights were recorded. The left biceps brachii and soleus muscles were fixed and resin-embedded for fibre number counting.

Body and muscle weights and lengths were significantly reduced in all restricted groups when compared to controls ($P < 0.05$). In general C > ER > TR > LR to the order of 15%, 35% and 50% reductions for ER, TR and LR respectively. Biceps fibre number was reduced in all restricted groups by 25%, 28% and 22% for ER, TR and LR groups ($P < 0.05$). Soleus fibre number was unaffected.

Thus, nutritional restriction in the first trimester resulted in a similar deficit in fibre number to that which resulted from restriction enforced throughout gestation. Restriction in the second and third trimesters also caused a fibre number deficit and the most severe reduction in fetal growth. These results may be explained by reduced myoblast proliferation and/or the effects of nutrition on placental growth.

Journal of Anatomy (1996) **189**: 230.

Muscle development in mice selected for large and small body weight. By S. C. BROWN and N. C. STICKLAND*. *Department of Biochemistry, Royal Holloway College, London and * Department of Veterinary Basic Sciences, The Royal Veterinary College, London*

Differences in fibre number may contribute up to two thirds of the divergence in muscle mass shown by mice selected for high (QL) and low (QS) body weight. Fibre number is determined around the time of birth in mammals and consequently myotube formation during the prenatal period is one of the single most important events influencing adult muscle size. The present study describes a quantitative assessment of muscle development in biceps brachii of the QL and QS from 15 to 19 d of gestation. Primary myotube number was higher in the QL relative to the QS, but the secondary to primary myotube ratio was lower. Nuclear density did not differ between the lines for either muscle at any stage. Although muscle girth at 19 d gestation was not significantly different between the lines, crown rump length and the length of the humerus showed a significant difference at this stage. Furthermore the differences between the strains in bone length were evident before any differences in the muscle. These observations lend support to the hypothesis that longitudinal bone growth acts as a 'pacemaker' for muscle growth.

Anat. Histol. Embryol. (1997) (in press)

Immunohistochemical localisation of Myod and Myogenin in developing fast and slow muscle fibres of the foetal mouse. **E. H Walters, N.C. Stickland and P.T Loughna.**
Department of Veterinary Basic Sciences, The Royal Veterinary College, London

Muscle development is describes as being a biphasic process; during the first phase multinucleate primary fibres are formed by the fusion of mononucleate myoblasts. This is followed by the formation of secondary fibres at which time the muscle becomes innervated. A group of basic helix-loop-helix transcription factors (bHLH) known as the myogenic regulatory factors have been shown to be integral to normal muscle development and differentiation. Their role has been investigated using mutants lacking one or more of the transcription factors and by utilising mRNA and protein localisation techniques including *in situ* hybridisation in whole embryos. MyoD has been shown to be expressed in proliferating, undifferentiated myogenic cells in vitro, while myogenin has been shown to be expressed only upon the induction of muscle differentiation. In postnatal muscle, re-innervation experiments have shown that myoD and myogenin are selectively expressed in fibres converting to fast and slow types respectively. Cellular localisation of these factors within muscle tissue has not been investigated in prenatal muscle. By using immunohistochemistry we have localised both myoD and myogenin at 14,16 and 18 days, while slow myosin heavy chain is present at 16 and 18 days gestation. At 18 days myogenin is only expressed in the secondary fibres that have a fast myosin phenotype, while myoD is expressed in the proportion of primary fibres that have a fast phenotype and in all secondary fibres. We therefore suggest that myoD is involved in the conversion of primary fibres that express slow myosin early in development, to primary fibres that express fast myosin heavy chain later in gestation. In conclusion this data suggests that these two bHLH proteins may play a crucial role in the determination of adult muscle fibre phenotype.

J. Anat. (1997) (in press)

Distribution of MyoD and myogenin in developing fast and slow muscle fibres of the foetal mouse. **E. H. WALTERS, N. C. STICKLAND, P. T LOUGHNA.** ***Department of Veterinary Basic Sciences, The Royal Veterinary College, London.***

The four myogenic regulatory factors (MRFs) MyoD, myogenin, Mrf-4 and Myf-5 are known to have vital roles in the proliferation and differentiation of myoblasts, which when fully differentiated form skeletal muscle. Myf-5 and MyoD are expressed in proliferating undifferentiated myoblasts whereas myogenin is expressed when muscle differentiation has been initiated. MRF-4 is usually expressed in mature skeletal muscle. The multiple roles of MRFs during muscle development suggests that each member may regulate a distinct group of genes, and that differential expression of MRFs may contribute to the diversity of the myogenic cells (Weintraub *et al*, *Science*, 251, 1991), namely the expression of fast and slow myosin isoforms. Work on rabbit muscle satellite cells has shown that MyoD and myogenin are present in slow satellite cells on day 6 of culture whereas fast satellite cells express MyoD from day 8 and, myogenin is expressed from day 12. Hughes *et al* (*Development*, 118, 1993) have shown in adult muscle that muscle fibres expressing fast myosin isoforms preferentially accumulate MyoD transcripts and slow fibres accumulate myogenin. The purpose of this investigation was to investigate whether MyoD and myogenin distribution was related to the expression of specific myosins in prenatal muscle. Pregnant Balb C mice were time mated and the foetuses removed at specific time points from E13 until E18. The foetuses were snap chilled and 10μm sections of the upper forelimb were cut on a cryostat. The sections were incubated with the primary antibody in 1% BSA in PBS for 2-3 days at 4°C. Anti-MyoD and Anti-myogenin polyclonal antibodies were used as well as an antibody to Slow MyHC. The sections were washed in 3 changes of PBS with 0.1% Tween 20. The FITC labelled secondary antibody in PBS with 1% BSA was applied for 3-5 hours at room temperature, and the staining was visualised using fluorescence microscopy. At day 13 Slow MyHC is present in newly formed primary fibres, MyoD and myogenin are present in the nuclei of cells in discreet areas of the limb bud, corresponding to those areas expressing slow MyHC. At 15 days Slow MyHC is present in all primary fibres while MyoD and myogenin are present in all muscle fibres. At 18 days slow MyHC is present in a proportion of muscle fibres whereas MyoD and myogenin are again present in all muscle fibres. From these observations we conclude that there is no evidence of MyoD or myogenin distribution being related to the development of specific fibre types, as determined by the distribution of slow MyHC.

2. Postnatal mammalian muscle including growth and adaptation

Anim. Prod. 1973, **16**: 135–146

A POSSIBLE INDICATOR MUSCLE FOR THE FIBRE CONTENT AND GROWTH CHARACTERISTICS OF PORCINE MUSCLE

N. C. STICKLAND AND G. GOLDSPINK

Muscle Research Laboratory, Department of Zoology, University of Hull

SUMMARY

Fibre number and fibre size were measured in the *flexor digiti V brevis* muscle and other muscles in pigs of different breeds. A large variation in fibre number (200%) was found among the breeds. However, the fibre number in the *flexor digiti V brevis* was closely correlated with fibre number of other muscles in the same animal. Fibre number and size measurements were also carried out on animals at different ages taken from a relatively pure breed of pig. Fibre size was found to increase to differing extents in different muscles, although the increase in the *flexor digiti V brevis* was almost the same as in the *longissimus dorsi*. Fibre number did not change during growth. On the basis of these findings it is suggested that the *flexor digiti V brevis* is a suitable indicator muscle for genetic experiments.

INTRODUCTION

RESEARCH into the mechanism of muscle fibre growth in laboratory animals has shown that the increase of muscle during growth is the result of an increase in the fibre size and is not due to an increase in the total number of fibres (Rowe and Goldspink, 1969). Although physiological conditions such as exercise and nutrition can affect the size of muscles (Goldspink, 1964), the number of fibres in the muscle is the more important factor in limiting ultimate muscle size. The total number of fibres in a given muscle is apparently genetically determined and fixed at or near birth (Luff and Goldspink, 1967). It would seem therefore that selection of animals for meat-bearing potential for breeding may have unwittingly been a selection of animals with greater fibre number (Luff and Goldspink, 1967). If a measurement of fibre number could be obtained from animals at a young age then this could be used for progeny testing, instead of waiting for the animals to mature. Staun (1968) suggested taking a small muscle sample by biopsy from the *longissimus dorsi*, while the area of the muscle is found by ultrasonics so that the total number of fibres in the muscle cross-section may be calculated. This biopsy method has been employed by Livingston, Blair and English (1966) who attempted to relate mean fibre diameter to indices of lean meat content of a range of bacon pig carcasses. Poor correlations were obtained and from this they concluded that more structural knowledge of the *longissimus dorsi* was required. The problem is that only relatively small samples of *longissimus dorsi* can be removed and so it is difficult to obtain a representative sample. Swanson, Kline and Goll (1965) showed large fibre size variation

between different locations in the *longissimus dorsi* muscle. The calculated fibre number for such a large and complex muscle can therefore only be a rough estimate. It seemed that a far better method would be to use a whole muscle for the biopsy sample and to carry out total fibre counts.

The purpose of this investigation was to find a suitable muscle for this biopsy technique which would have a fibre number representative of the fibre number of other muscles in the same animal. The feasibility of the use of this method in genetic experiments has also been investigated by comparing the fibre number in various breeds of pigs.

A muscle biopsy method of progeny testing could really only work successfully if the fibre number does not change with growth. Therefore a growth study was carried out in which fibre number and size were measured in the indicator muscle and two meat muscles of a reasonably pure breed of pig.

MATERIAL AND METHODS

Microanatomy of muscles. Six possible indicator and comparison muscles were dissected out from several pigs. They were fixed in 2·5% glutaraldehyde at 4°C for 18 hr, washed in phosphate buffer (pH 7·2) for 6 hr and left in 30% nitric acid for two days (pulling fibres slightly apart after one day) and washed again. It was then possible to tease the fibres in 50% glycerol using fine needles. This procedure (Williams and Goldspink, 1971) enabled the way in which the fibres run within the muscles to be determined. Of the muscles studied four were found to be suitable for future investigation and one suitable as an indicator muscle.

Histology. The four different anatomical muscles were removed from several pigs which were obtained from local farms and were usually less than 4 weeks old. The muscles were removed entire, cleared of connective tissue and weighed. They were then fixed in 2·5% glutaraldehyde, dehydrated and embedded in Paraplast. Glutaraldehyde was chosen because of its quick penetrating properties which are needed when using reasonably large muscles. All muscles were removed post-rigor, so no further fixing of the length after removal, was necessary. Transverse sections were cut at 6 μ on a Beck rotary microtome, stained in Heidenhain's iron haematoxylin and fibre counts made at about five levels per muscle. Counting was accomplished by projecting a complete section from a microscope on to white paper and counting every fibre in the section using an electronic pen counter. In some cases complete serial sections were cut in order to obtain more detailed knowledge of the internal architecture of the muscles, so that the level where the maximum number of fibres were included in the transverse section could be established. On some sections fibre diameter was also measured. This was carried out using an ocular micrometer scale in the eyepiece of the microscope. Histological processing invariably causes shrinkage of the fibres and the method used here was no exception. In this case it was found that the histological processing caused the fibres to shrink by approximately one-third. The diameter measurements were thus corrected for this, although the measurements were used in a comparative way and therefore the exact shrinkage was not important.

Growth study. The muscles for this investigation were all obtained from pigs used in a study at the Department of Anatomy of the Royal (Dick)

School of Veterinary Studies, Edinburgh. The pigs were all females from a selected herd at Cockle Park Farm, Newcastle.

The indicator muscle (*m. flexor digiti V brevis*), *sartorius* muscle and a slice of the *longissimus dorsi* (at the thoracicolumbar junction) were removed from each pig (post-rigor) and 6μ transverse sections were prepared from the belly of the muscle. The weights of the muscles were noted immediately after dissection together with other various carcass parameters including total muscle weight and total fat weight. The transverse sectional area of the *longissimus dorsi* muscle (at thoracicolumbar junction) was determined from the slide using a planimeter following fixation and sectioning of the tissue. Hillers (1970) showed that planimetry was slightly more accurate in measuring *longissimus dorsi* areas than grid methods. For the small indicator muscle, total fibre counts were carried out as described above. However, for the larger *sartorius* and *longissimus dorsi* muscles a grid was superimposed on to the projected image so that the number of fibres per unit area could be calculated. This was multiplied by the transverse sectional area of the entire section on the slide to obtain the total number of fibres in the muscle. Fibre diameter measurements were also taken from these muscles at different stages of growth.

RESULTS

Feasibility of indicator muscle. The four muscles found to be convenient to use were

M. flexor digiti V brevis	Indicator
M. extensor digitalis communis, deephead	Comparisons
M. extensor digiti I longus	Comparisons
M. rhomboideus capitis	Comparisons

These muscles were chosen because their fibres run longitudinally from origin to insertion (determined by teasing in glycerol) and all the fibres are included in a transverse section through the belly of the muscle. They were also small enough so that counting all the fibres was feasible. The *m. flexor digiti V brevis* was chosen as the indicator muscle because of its very small size and its easily accessible position (Figure 1): after making a suitable incision in the skin the distal tendon of this muscle, which overlies the *m. abductor digiti V*, can be lifted up in this region and the muscle dissected up to its proximal attachment on the accessory carpal. Another important factor considered was that removal of the indicator muscle should not unduly impair the animal's locomotion if it were to be used as a routine biopsy technique. Also it should preferably be of no commercial importance as its removal must not detract from the commercial value of the carcass.

The results of the teasing investigations and the serial sectioning of the muscles are shown in Figure 2. These investigations provided information which enabled sections to be made which contained all the fibres of the muscle.

The results of the fibre counts of indicator muscles from all the individual pigs used are shown in Table 1. In some cases fibre counts were made on the indicator muscle from both sides of the animal to ensure bilateral symmetry and these results are summarized in Table 2. The number of fibres in the indicator muscle was then plotted against numbers in the three comparison

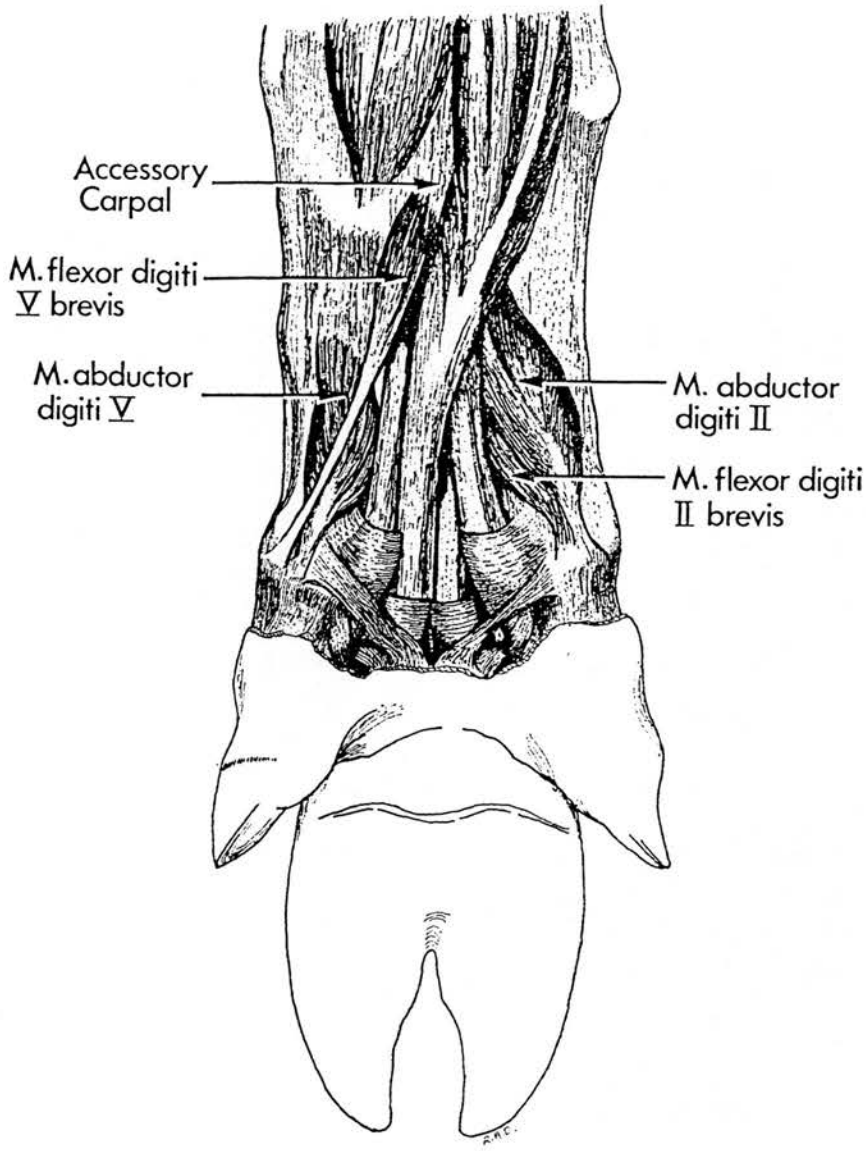

FIG. 1. Anatomical location of indicator muscle.

muscles and are shown in Figure 3. Each point on the graphs represents one animal and the lines given are the computed regression lines. The correlation coefficients of fibre number in the indicator muscle with fibre numbers in the comparison muscles were all significant at the 0·001 probability level (Table 3). The other correlations of muscle weights with body weight, etc. were also significant except the correlations involving the weight of indicator muscle itself (Table 3). This is probably due to the fact that the muscle is very small and so any connective tissue present has a relatively large effect on its weight. Certainly, younger animals tended to have relatively more connective tissue

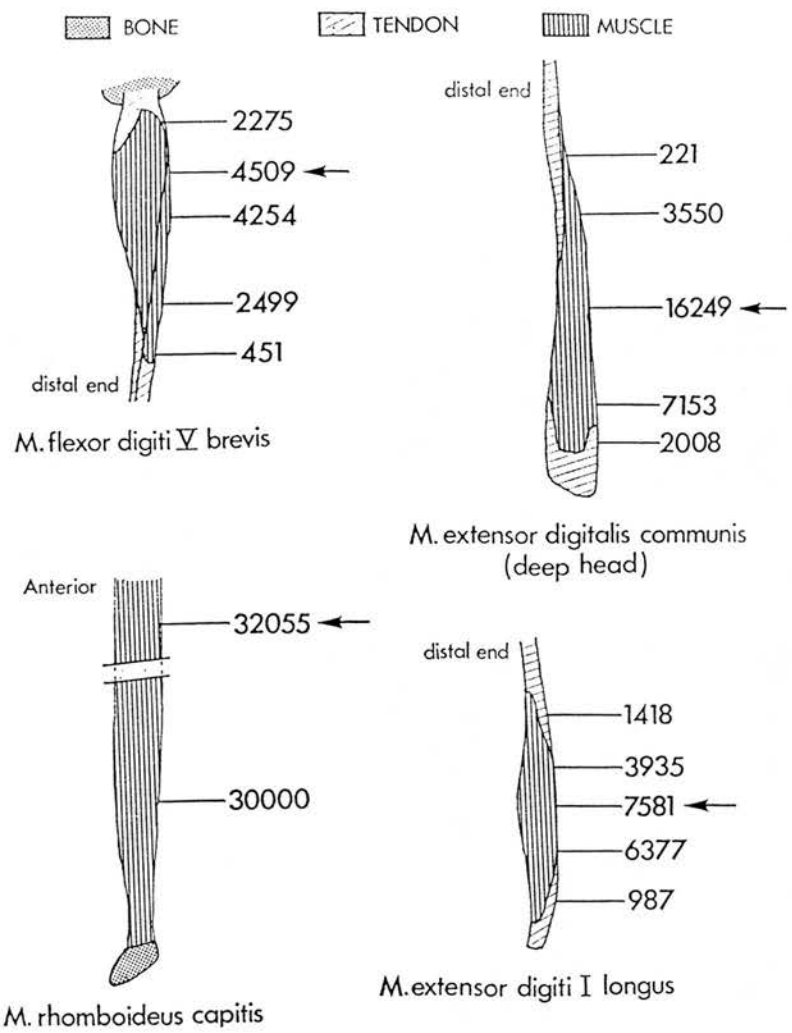

FIG. 2. Micro-anatomy of muscles.

which could not be removed from the muscle without damaging the fibres.

The variation of fibre number in the indicator muscle between different breeds is shown in Table 1. From this Table it will be seen that the fibre number varied by about 200% (2300 to 6500). There was considerable variation within any particular breed, probably due to differences among herds of the same breed; for example in the pure Large White pigs, W_1, W_2 and W_3 (3702, 3839 and 3863 respectively) were from one herd, W_4 (6804) and W_5 (6008) from another herd. The first herd had in fact been closed to outside blood for several years. At 18 days of age the average fibre diameter (for indicator muscle) of the first herd was 19·6 $\mu \pm 0{\cdot}37$; that of the second was 14·0 $\mu \pm 0{\cdot}21$. (Differences were significant at $P < 0{\cdot}001$.) Hence, in this case, it appears that large fibre number was associated with smaller fibres and vice versa.

TABLE 1

Muscle fibre counts

No.	Sow	Boar	*Flexor digiti V brevis* Indicator
1	Landrace × Large White	Camborough	4509
2	Large Black × Landrace	Camborough	4171
3	Landrace × Large Black	Landrace	4602
4	Large Black × Large White	Large White	6049
5	Landrace × Large White	Landrace	4675
6	Large Black × Large White	Camborough	3934
7	Landrace × Large Black	Camborough	5007
8	Large White × Large Black × Landrace	Landrace	4448
9	Large White	Landrace	5112
10	Welsh	Camborough	2372
11	Landrace × Large Black × Large White	Landrace	6520
12	Large White	Landrace	5004
13	Welsh	Landrace	3265
14	Large White × Landrace	Large White	5190
15	Large White × Landrace	Large White	5684
16	Welsh	Large White	3180
W_1	Large White	Large White	3702
W_2	Large White	Large White	3839
W_3	Large White	Large White	3863
W_4	Large White	Large White	6804
W_5	Large White	Large White	6008
L_1	Landrace	Landrace	3452
L_2	Landrace	Landrace	3438
L_3	Landrace	Landrace	3279
S	Saddleback	Saddleback	6013
C_1	Camborough	Camborough	3365
C_2	Camborough	Camborough	2805
C_3	Camborough	Camborough	3602
P_1	Pietrain	Pietrain	3675
P_2	Pietrain	Pietrain	4711
P_3	Pietrain	Pietrain	3721
P_4	Pietrain	Pietrain	4729

TABLE 2

Fibre counts from indicator muscle of both sides

Pig. no.	Left muscle	Right muscle	Difference
1	4509	4584	Approx. 2%
2	4171	4381	Approx. 5%
3	4602	4712	Approx. 3%
4	6049	5990	Approx. 1%
6	3934	4186	Approx. 6%

Diameters of fibres were measured in the indicator muscle for four Large White and four Pietrain pigs. The results were as follows:

	Large White	Pietrain
Carcass weight (kg)	$8{\cdot}23 \pm 1{\cdot}03$	$8{\cdot}31 \pm 0{\cdot}34$
Fibre diameter (μM)	$19{\cdot}75 \pm 1{\cdot}66$	$27{\cdot}53 \pm 1{\cdot}77$

(The difference in the diameters is significant at $P < 0{\cdot}02$.)

Growth study. The muscles used were taken from 18 pigs varying in live weight from about 1 to 64 kg (about 2 to 200 days of age). The weight of the

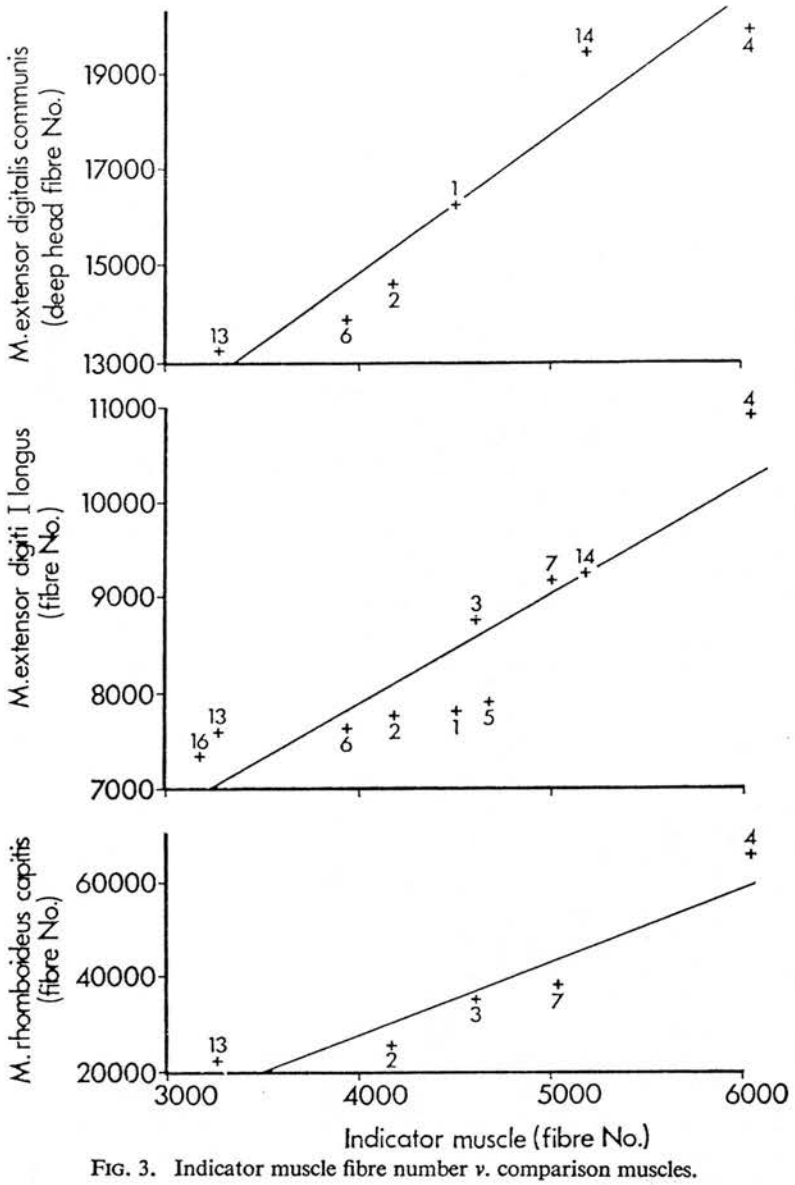

FIG. 3. Indicator muscle fibre number *v.* comparison muscles.

indicator muscle was highly correlated with the total body muscle weight ($r = 0{\cdot}8394$, $P < 0{\cdot}001$).

The changes in fibre diameter associated with increase in total muscle weight are shown in Figure 4. Correlation analyses were carried out between indicator muscle fibre diameters and various weight parameters (Table 3). The significance of the correlation coefficients showed that larger fibres were associated with greater muscle mass.

In Figure 5 the mean fibre area of the *longissimus dorsi* is plotted against transverse sectional area of the same muscle; there was a good linear relationship between fibre area and muscle area.

TABLE 3

Correlations between various traits†

	r	No. (pairs)	P
Fibre no. 1 *v.* fibre no. 2	0·9856	6	***
Fibre no. 1 *v.* fibre no. 3	0·8933	10	***
Fibre no. 1 *v.* fibre no. 4	0·9291	5	*
Weight 1 *v.* weight 2	0·2438	9	NS
Weight 1 *v.* weight 3	0·4148	9	NS
Weight 2 *v.* weight 3	0·8485	9	***
Weight 1 *v.* body weight	0·4468	10	NS
Weight 2 *v.* body weight	0·9343	9	***
Weight 3 *v.* body weight	0·7606	10	***
Weight 4 (cm) *v.* body weight	0·8330	4	*
Fibre diameter 1 *v.* age	0·7824	18	***
Fibre diameter 1 *v.* carcass weight	0·7959	18	***
Fibre diameter 1 *v.* total muscle weight	0·8052	18	***
(Fibre diameter)^2 1 *v.* total muscle weight	0·8028	18	***

†1 = *M. flexor digiti V brevis*—indicator,
2 = *M. extensor digitalis communis*, deep head,
3 = *M. extensor digiti I longus*,
4 = *M. rhomboideus capitus*.

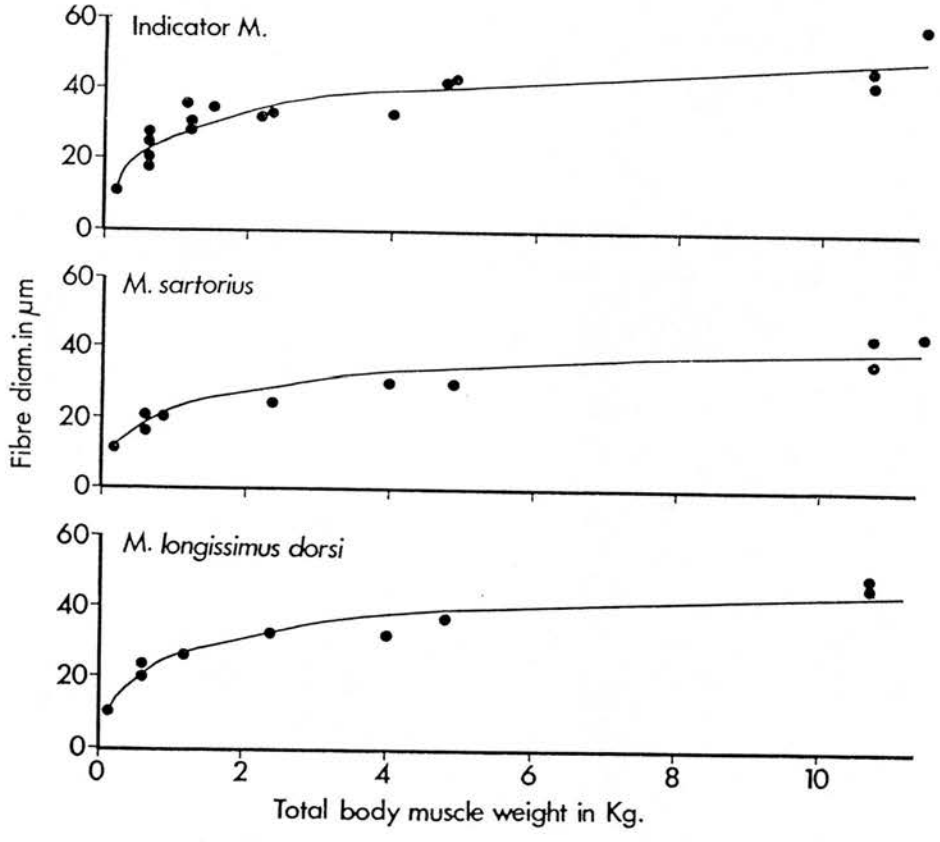

FIG. 4. Fibre diameter changes with growth.

The results of the changes in fibre number with growth are shown in Figure 6 for the indicator muscle, *m. sartorius*, and *m. longissimus dorsi* respectively. The computed regression lines for these plots were not significantly different from the horizontal which strongly indicates that fibre number did not change during growth.

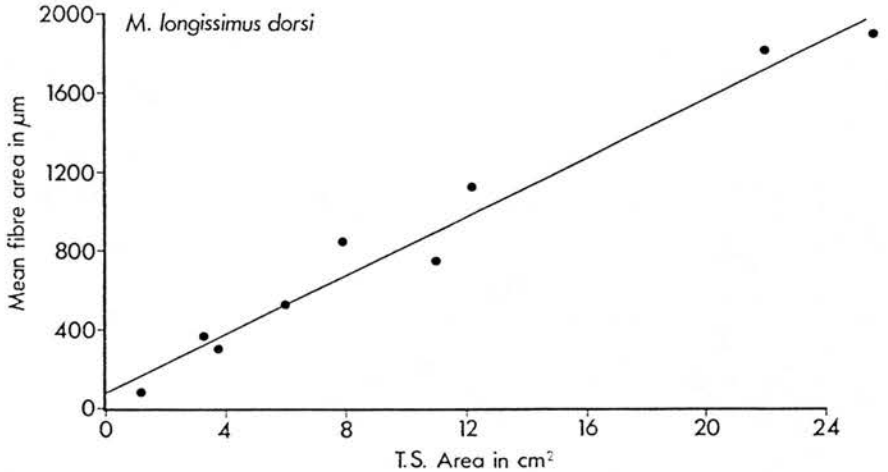

FIG. 5. Changes in mean fibre cross-sectional area with changes in muscle cross-sectional area.

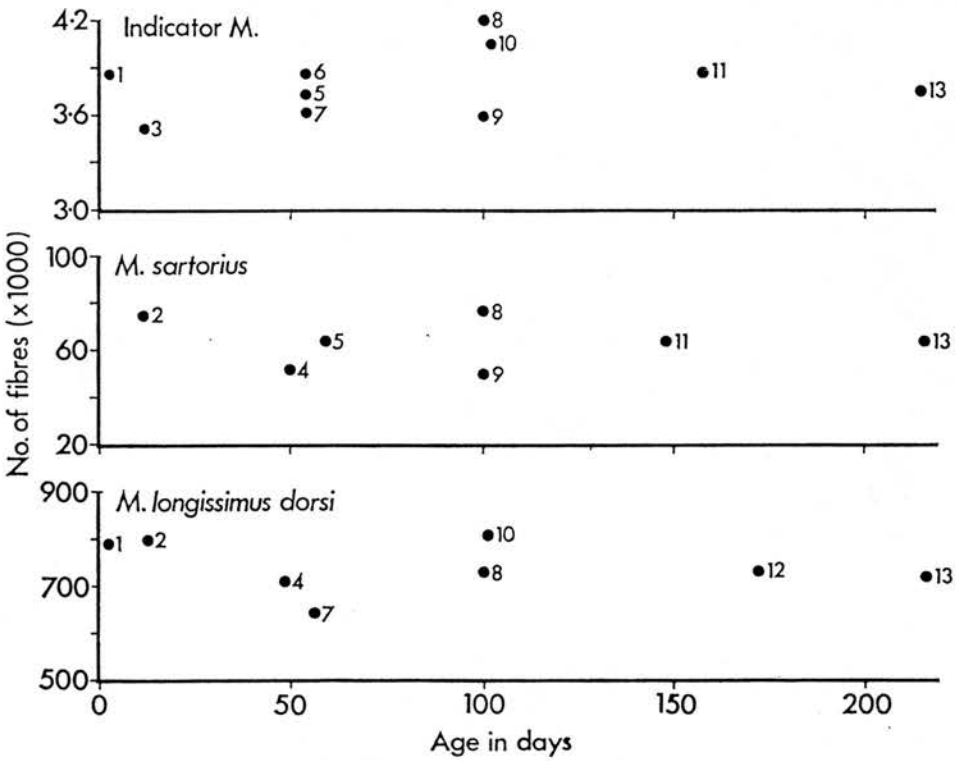

FIG. 6. Changes in fibre number with age.

DISCUSSION

It would appear that the indicator muscle used was representative of other muscles in the body, with respect to fibre number. The fact that the three 'comparison' muscles were from different regions of the body (fore limb, hind limb and neck region) ensured that the musculature as a whole was well represented. The fibre counts of the indicator muscle from each side varied from 1% to 6% with each given animal; this demonstrates the high degree of bilateral symmetry. This small variation between contralateral muscles is negligible compared with interbreed variations of up to 200%.

The large variation in fibre number in the indicator muscles studied should make it possible to construct breeding programmes to achieve high fibre number, especially if a routine method of fibre number counting can be developed using automatic counting instrumentation. Within a breed it appeared that large fibre number was associated with smaller fibres and *vice versa*. Staun (1968) found that the total number of fibres was negatively correlated with the fibre diameter, that is to say the larger the number the thinner the fibres. It is interesting to note that 'herd' differences in fibre number have also been found in other animals. Luff and Goldspink (1967) found fibre number differences in two strains of mice bred from the same original stock. Also Walker, Burd and Pull (1972) found significant differences in fibre number among stocks of North Sea herring.

As far as work on fibre size is concerned, significant differences between breeds have been reported by Mauch and Marinesco (1934), Glebina (1952), and Lawrie and Gatherum (1964). In the present study significant differences ($P < 0{\cdot}02$) were found between the two breeds on which measurements were made, the Large White and Pietrain. Dumont and Schmitt (1970) found that the Pietrain had larger fibres than Large Whites in certain muscles. Staun (1963) associated large fibres of the Pietrain with poor meat quality. Possibly, therefore, increased fibre number might result in improvements in meat quality as well as quantity.

In the growth study, the fibre diameters of the indicator and the two meat muscles showed similar increases with increase in total body muscle weight (Figure 4). The indicator muscle and the *m. longissimus dorsi* showed fibre diameter increases from about 10 μ at 0·2 kg total body muscle weight to about 48 μ at 11 kg. The fibre diameters of *m. sartorius* appeared to increase to a lesser extent from about 11 μ at 0·2 kg to 38 μ at 11 kg total body muscle weight. This difference in fibre size between different muscles of the same animal has been demonstrated by several authors (Hammond and Appleton, 1932—sheep; Joubert, 1956—sheep; Staun, 1963—pigs). This difference is probably due to the different workloads on the muscles, which therefore develop to different extents (Rowe and Goldspink, 1969).

The highest correlation involving fibre diameter (Table 3) was its squared value with total body muscle weight ($r = 0{\cdot}8380$, $P < 0{\cdot}001$), though the relationship was still not linear. If fibre cross-sectional area is plotted against muscle cross-sectional area (Figure 5) the variation due to changing fibre length is not, of course, involved, and it is for this reason that a good linear relationship and better correlation are obtained ($r = 0{\cdot}9835$, $P < 0{\cdot}001$). In fact, for *m. longissimus dorsi*, the mean fibre area increased by 2·2 times whereas the weight of the muscle increased by 8·8 times during the period studied. This would indicate an increase in muscle length of about 4 times

(assuming the *m. longissimus dorsi* is uniform throughout its length) in agreement with actual results for thoracic plus lumbar length increases. Increase in muscle length, as well as muscle girth, must therefore contribute very significantly to increase in weight of this muscle.

Perhaps the most important finding, as far as this study is concerned, is that the fibre number in the three different anatomical muscles did not increase during growth.

It would seem therefore that the indicator muscle could be removed at an early age and the number of fibres counted. As the number of fibres does not change with age, it would indicate the fibre number of other muscles, because of the close correlations found. Since fibre number is related to muscle mass, a muscle biopsy technique can presumably be used for progeny testing with particular reference to increasing muscle size. Using this method, herds with higher fibre number could be established. The selection of animals for breeding based on cellular characteristics obviously requires more intensive study.

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REFERENCES

DUMONT, B. L. and SCHMITT, O. 1970. [Comparative study of muscular structure in pigs of Large White and Pietrain breeds.] *Ann. Génét. Sél. Anim.* **2** (4): 381–393.

GLEBINA, E. L. 1952. Changes in the muscle tissue of pigs due to cross-breeding. *Anim. Breed. Abstr.* **21**: No. 299.

GOLDSPINK, G. 1964. The combined effects of exercise and reduced food intake on skeletal muscle fibres. *J. Cell. Comp. Physiol.* **63**: 209–216.

HAMMOND, J. and APPLETON, A. B. 1932. Study of the leg of mutton. Part V. In *Growth and the Development of Mutton Qualities in the Sheep.* Oliver and Boyd, London.

HILLERS, J. K. 1970. Comparing three methods of measuring longissimus area. *J. Anim. Sci.* **31**: 843–845.

JOUBERT, D. M. 1956. An analysis of factors influencing post natal growth and development of the muscle fibre. *J. agric. Sci., Camb.* **47**: 59–102.

LAWRIE, R. A. and GATHERUM, D. P. 1964. Studies on the muscles of meat animals. V. Inter- and intra-litter differences in the composition of longissimus dorsi muscles from pigs. *J. agric. Sci., Camb.* **62**: 381–390.

LIVINGSTON, D. M. S., BLAIR, R. and ENGLISH, P. R. 1966. The usefulness of muscle fibre diameter in the studies of lean meat content of pigs. *Anim. Prod.* **8**: 267–273.

LUFF, A. R. and GOLDSPINK, G. 1967. Large and small muscles. *Life Sci.* **6**: 1821–1826.

MAUCH, A. and MARINESCO, J. 1934. Comparison of the flesh of Mangulica, Large White and Lincoln breeds and their crosses. *Anim. Breed. Abstr.* **3**: 50.

ROWE, R. W. D. and GOLDSPINK, G. 1969. Muscle fibre growth in five different muscles in both sexes of mice. I. Normal mice. *J. Anat.* **104**: 519-530.

STAUN, H. 1963. Various factors affecting number and size of muscle fibres in the pig. *Acta Agric. scand.* XIII: 293–322.

STAUN, H. 1968. [Diameter and number of muscle fibres and their relation to meatiness and meat quality in Danish Landrace pigs.] 366 Beretn. fra forsøgslab. København., p. 121.

SWANSON, L. A., KLINE, E. and GOLL, D. 1965. Variability of muscle fibre size in bovine longissimus dorsi. *J. Anim. Sci.* **24**: 97–101.

WALKER, M. G., BURD, A. C. and PULL, G. A. 1972. The total numbers of white skeletal muscle fibres in cross section as a character for stock separation in North Sea Herring. *J. Cons. int. Explor. Mer.* **34**: 238-243.

WILLIAMS, P. E. and GOLDSPINK, G. 1971. Longitudinal growth of striated muscle fibres. *J. Cell. Sci.* **9**: 751–767.

(*Received* 30 *March* 1972)

Anim. Prod. 1975, **21**: 93–96

A NOTE ON PORCINE SKELETAL MUSCLE PARAMETERS AND THEIR POSSIBLE USE IN EARLY PROGENY TESTING

N. C. STICKLAND† AND G. GOLDSPINK

Muscle Research Laboratory, Department of Zoology, University of Hull

SUMMARY

An 'indicator muscle' (*m. flexor digiti V brevis*) was removed in its entirety from several Large White and Landrace pigs of varying fat depth (measured over the eye-muscle area). The total muscle fibre number was measured in transverse sections for each muscle. Significant inverse relationships were found between this fibre number and fat depth measurements. These and other results suggest that fibre number is related to lean meat content. Fibre number was also shown to be highly correlated with fibre density and as thinner fibres (i.e. higher fibre density) are associated with more tender meat, fibre number may be associated with quality as well as quantity of meat.

IN a previous paper (Stickland and Goldspink, 1973) a muscle was described in the pig which was indicative of other muscles in the animal with respect to muscle fibre number. This muscle, termed the indicator muscle, was the *m. flexor digiti V brevis*. The possibility was suggested of using this muscle as an index for early progeny testing as well as for performance testing.

Selection for animals with meat-bearing potential may unwittingly be a selection for those animals with more muscle fibres (Luff and Goldspink, 1967; Staun, 1968). Miller (1970) showed that the number of fibres in the *longissimus* muscle is more related to muscle mass than is the size or type of fibres. This was in agreement with Livingston, Blair and English (1966) who found no correlation between fibre diameter and lean meat content. Therefore, in this investigation it was decided to pay particular attention to muscle fibre number, using the indicator muscle, and to relate this to conventional methods of carcass assessment.

The animals used were from the Meat and Livestock Commission's Pig Testing Stations, were reared under standard conditions, and included Large White and Landrace breeds of pig. Both young adult females (gilts) and castrated males were used, but as sex does not seem to affect muscle fibre number in pigs (Staun, 1963; Stickland, 1973) this variable was not considered here. Within each breed there was a range of fat and lean pigs, classification being based upon C+K fat depth measurements (explained below).

Left forefeet were taken from all these animals at the time of slaughter and, after removal of the skin, were placed in 2·5% glutaraldehyde fixative in phosphate buffer (pH 7·2). The indicator muscle was removed in its entirety from all these animals and processed as described previously (Stickland and Goldspink, 1973) to obtain 7 μ transverse sections through the 'belly' of

† Present address: Department of Veterinary Anatomy and Histology, University of Nairobi, PO Box 30197, Nairobi, Kenya.

the muscle. The total number and density of the muscle fibres in these sections were measured.

Various carcass parameters taken from the left side were used in this study. These were as follows: weight of *M. psoas major*, the greatest width of the 'eye muscle' *M. longissimus* (A), the greatest depth of the 'eye muscle' at right angles to A (B), the thickness of fat and skin immediately over B at right angles to skin (C), and the thickness of fat and skin measured at the dorso-lateral corner of the 'eye muscle' at right angles to skin (K). Measurements A, B, C and K were taken from a cut surface at the thoraco-lumbar junction. Measurements A and B were used to calculate the 'eye-muscle' index, X, whereby

$$X = \pi \frac{A.B}{4}$$

This product gives the cross-sectional area for an ellipse to which the 'eye muscle' approximates and is the same formula used by Joubert (1956) to estimate 'eye-muscle' cross-sectional area in sheep. C and K were added together in this study to provide a C+K fat depth index.

TABLE 1

Correlation coefficient results

Parameters correlated	Large White‡	Landrace§
Fibre number† and 'C+K'	−0·464*	−0·614*
psoas weight	0·640**	0·198
longissimus 'X' ('area')	0·306	0·245
fibre density (no. per unit TS area)	0·700***	0·520
'C+K' and *longissimus* 'X'	−0·640**	−0·474
Partial correlations		
Fibre number† and 'C+K' excluding CDW‖	−0·461*	−0·584

† Total number of muscle fibres in the indicator muscle (*m. flexor digiti V brevis*).
‡ 21 animals.
§ 11 animals.
‖ CDW Cold dead weight.

The correlation coefficients and partial correlation coefficients using the results from all the pigs are shown in Table 1. The relationships between fibre number and C+K is shown graphically in Figure 1 for both breeds.

It is already generally accepted that C+K fat depth measurements are a good indication of a carcass with respect to lean meat content. This is reflected in the negative correlation coefficient between 'eye-muscle area' (X) and C+K values (Table 1). Hence, the finding that there is a significant negative correlation between muscle fibre number and C+K values is considered to be important. The other results in Table 1 also support the fact that fibre number is related to the carcass grade. The correlations, which were not significant (note especially the Landrace), presumably require more animals for the relationship to be established. The equations for the re-

gression lines shown in Figure 1 are given below, together with the SE for an estimation of Y(C+K fat depth) on X(fibre number):

Large White $Y = -0{\cdot}3027.X + 53{\cdot}51$ $Sy.x = \pm 12{\cdot}02$
Landrace $Y = -0{\cdot}3355.X + 46{\cdot}60$ $Sy.x = \pm 5{\cdot}29$

From the computed regression lines of Figure 1, for any given fat depth, the Large White pigs have a higher fibre number than the Landrace, although the slopes of the lines do not differ significantly. This fibre number difference could be due to a breed effect (Stickland, 1973) although the difference in mean fibre number is not significant here. The insignificant difference may be caused by the large variance in the Large White fibre number compared to the Landrace (F ratio is significant at $P < 0{\cdot}01$). It would seem, however, that some of this variation can be related to carcass measurements such as fat depth. A relationship between fibre number and fat content within a

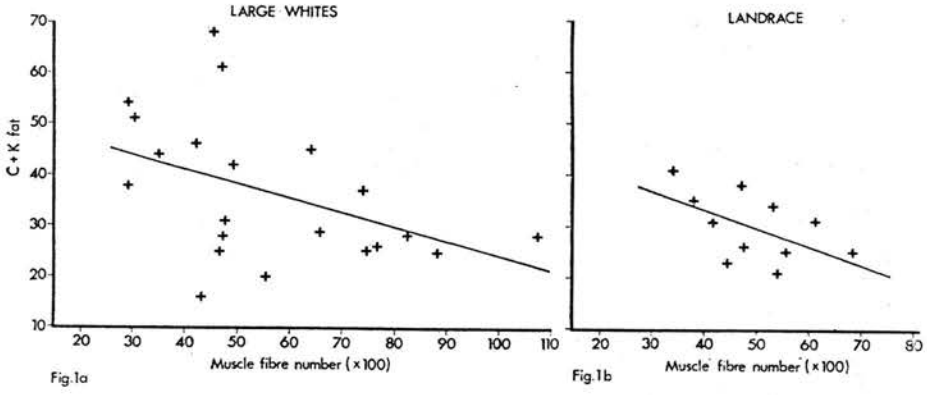

FIG. 1. Relationship between muscle fibre number and carcass fat measurements (C+K).

breed has also been shown by Staun (1963) who found no significant difference in 'eye-muscle' fibre number between Yorkshire and Duroc pigs, whereas he did find a difference ($P < 0{\cdot}05$) between high and low fat lines within the Duroc race.

The highly significant correlation for Large White pigs between total muscle fibre number and fibre density (related to fibre size) (Table 1) is also interesting. It is important in that other workers, including Carpenter, Kauffman, Bray, Briskey and Weckel (1966), have shown that increased fibre size is related to a decrease in tenderness. It would seem, therefore, that selection for high fibre number content may increase the quality of the meat as well as the quantity.

As fibre number in the indicator muscle does not change with age (Stickland and Goldspink, 1973) it is suggested that this muscle could possibly be used as a means of early progeny testing. If the muscle is removed at an early age its fibre number content could be used in prediction equations such as those shown above.

However, the relationships shown in Figure 1 for Large White and Landrace suggest that only about 22% and 27% respectively of the variation (r^2) in C+K was explained by fibre number. This may well be because only phenotypic correlations were investigated. Individual food intake was not

recorded and could easily affect C+K measurements as different pigs will have different growth rates, feed conversion efficiencies, etc. The next step should be to investigate the genetic correlation using animals with a controlled and recorded environment. Such a correlation may be higher and provide a more accurate prediction of the animal's genetic potential. The present prediction equations are not accurate for progeny testing but provide the basis for future work.

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REFERENCES

CARPENTER, Z. L., KAUFFMAN, R. G., BRAY, R. W., BRISKEY, E. J. and WECKEL, K. G. 1966. Factors influencing quality of pork. A. Histological observations. *J. Food Sci.* **28**: 467–471.

JOUBERT, D. M. 1956. An analysis of factors influencing post natal growth and development of the muscle fibre. *J. agric. Sci., Camb.* **47**: 59–102.

LIVINGSTON, D. M. S., BLAIR, R. and ENGLISH, P. R. 1966. The usefulness of muscle fibre diameter in studies of the lean meat content of pigs. *Anim. Prod.* **8**: 267–275.

LUFF, A. R. and GOLDSPINK, G. 1967. Large and small muscles. *Life Sci.* **6**: 1821–1826.

MILLER, L. R. 1970. Influences on porcine muscle fibre attributes. *Diss. Abstr.* **30**: No. 4859-B.

STAUN, H. 1963. Various factors affecting number and size of muscle fibres in the pig. *Acta Agric. scand.* **XIII**: 293–322.

STAUN, H. 1968. [Diameter and number of muscle fibres and their relation to meatiness and meat quality in Danish Landrace pigs.] *No.* 366. *Beretn. fra Forsøgslab.*, København.

STICKLAND, N. C. 1973. The growth and development of skeletal muscle in pigs. *Ph.D. Thesis, Univ. Hull.*

STICKLAND, N. C. and GOLDSPINK, G. 1973. A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Anim. Prod.* **16**: 135–146.

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Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscle of pigs

By N. C. STICKLAND*

Zoology Department, University of Hull, Hull

AND ELSIE M. WIDDOWSON

Department of Investigative Medicine, University of Cambridge, Cambridge CB2 1QN

AND G. GOLDSPINK

Zoology Department, University of Hull, Hull

1. Measurements have been made of the size and number of muscle fibres and number of nuclei in a small indicator muscle (m. flexor digiti V brevis) in the fore-foot of the pig. Well-nourished, 10-d-old and 1-year-old animals were studied, as well as 1- and 2-year-old animals that were severely energy-deficient and protein-deficient.

2. The normal 1-year-old animals had much larger muscle fibres, with more nuclei, than the pigs in any of the other groups.

3. There were no significant differences between the numbers of fibres in the muscles of pigs in any of the four groups.

4. There was a significant difference between the number of fibres in the muscles of pigs coming from different litters, irrespective of their dietary history after birth. This suggests that the number of fibres is determined genetically before birth, and all that can take place after birth is an alteration in size.

Research on the skeletal muscle of laboratory animals has shown that different nutritional states can affect the size of a muscle by altering the size of the fibres within it (Goldspink, 1964), although the total number of fibres does not change. In farm animals, McMeekan (1940, 1941) found that feeding had a considerable effect on the diameter of muscle fibres in pigs, and Joubert (1956) reported that starvation decreased the diameter of the muscle fibres in sheep. Staun (1963) studied the effects of various levels of dietary protein on the skeletal muscle of pigs and found changes in the diameter of muscle fibres. Johnson (1971), working with bovine muscle, also found that fibre size was reduced by restricted nutrition. However, little accurate work has been done on the effect of nutrition on the total number of fibres in the muscles of farm animals. In a previous paper (Stickland & Goldspink, 1973) a small indicator muscle was described (m. flexor digiti V brevis) which behaved as did other muscles in the same pig. In particular the number and size of fibres in this muscle changed in parallel with those in the longissimus dorsi muscle. This small muscle can be removed entire and total muscle fibre counts can be made. It was decided, therefore, to use this indicator muscle in a study of skeletal muscle from pigs given low-energy and low-protein diets. The dietary deprivation in these experiments was much more

* Present address: Department of Veterinary Anatomy, University of Nairobi, PO Box 30197, Nairobi, Kenya.

severe than that used in previous studies. Measurements were made of the number and size of the fibres, together with the number of nuclei in the muscles, and their DNA content.

MATERIALS AND METHODS

Animals and diets. Muscle samples were taken from Large White pigs from five litters which were being used in studies of the effects of severe energy and protein deficiencies. Thirteen piglets were fed on severely restricted amounts of the stock diet, containing 180 g protein/kg, from 10 d of age so that at the end of 1 year their body-weights were only about 3 % of normal (McCance, 1960). Six piglets were killed at that time and the remaining seven piglets were maintained on the same diet for a second year; they were allowed to grow from 6 kg at 1 year of age to 9–11 kg at 2 years of age. Ten 10-d-old piglets were given the same amount of the stock diet together with as much sugar or fat as they would eat (McCance, 1968). As they became older and more accustomed to the diet they ate more sugar or fat, so that by 10 weeks of age they were eating enough to reduce the protein content of their diet from the normal 180 to about 60 g/kg. These animals gained more weight than the energy-deficient ones and at the end of 1 year weighed 13–19 kg, some 7–8 % of their 'expected' weight at that age. Five piglets were killed at this stage and the other five were maintained on the diet for a second year, by which time they weighed 16–29 kg.

Five normal piglets aged 10 d were killed and their muscles examined. This represented the situation at the start of the period of deprivation. Five pigs, reared normally, were killed at weights of about 200 kg, at 1 year of age.

The 10-d-old pigs and the energy- and protein-deficient ones were killed by injection of a lethal dose of Nembutal (Abbott Laboratories Ltd, Queenborough, Kent) directly into the heart. The older control pigs were slaughtered in the usual manner at the abattoir. The required muscles were removed and examined as soon after death as possible.

Histological examination. The indicator muscle, m. flexor digiti V brevis, from the left fore-foot was cleaned (but tendons were not cut) and fixed in situ for up to 30 min using glutaraldehyde solution (25 g/l) adjusted to pH 7·2 with phosphate buffer (41·5 ml $NaH_2PO_4.H_2O$ solution (45·2 g/l) plus 7 ml NaOH solution (50·4 g/l); pH adjusted to 7·2 with additional NaOH). The muscles were then dissected out, freed from any residual connective tissue and fat, and returned to the fixative solution. The muscles were dehydrated in ethanol, cleared in cedar-wood oil, embedded in Paraplast wax (Sherwood Medical Industries Ltd, Crawley, Sussex), and transverse 7 μm sections cut on a Beck rotary microtome (Beck Instruments Ltd, Watford, Herts.). The sections were stained in Delafield's haematoxylin and eosin, and projected onto white paper using a projection microscope (E. Leitz (Instruments) Ltd, London W1N 8BB). The total number of muscle fibres was counted using an electronic pen counter, and the total number of nuclei visible in the transverse section was also counted.

The total number of nuclei in each muscle was calculated in the following way. If

n is the apparent number of nuclei counted in the muscle transverse section, then the real number (N) of whole nuclei in the section $= \frac{T}{T+D} \times n$ (Abercrombie, 1946)

In this instance, thickness of the sections (T) was about the same as the diameter of the nuclei (D) in the long axis. This was found by measuring them in a longitudinal section of pig muscle; the maximum length was found to be about 8 μm. It may be added here that Strobykina (1970) found that lengths of nuclei remained the same throughout the growth of Large White pigs. Therefore, it seemed reasonable to assume that the length of the nuclei would be the same in all the sections and approximately equal to their thickness. Therefore, $N = \frac{n}{2}$.

The approximate volume (V) of the indicator muscle was calculated using the formula:

$$V = \left(A \times \frac{l}{3}\right) + \left(\frac{A}{3} \times \frac{2l}{3}\right),$$

where A is the area of section taken in the 'belly' of the muscle, from which nuclei counts were made; l is the length of the muscle in its wax block; $A \times \frac{l}{3}$ is the volume of the cylindrical part of the muscle; $\frac{A}{3} \times \frac{2l}{3}$ is the volume of the tapering part of the muscle, corresponding to the volume of a cone.

It follows that the total number of nuclei in the muscle is then: $\frac{N}{AT} \times V$. The concentration of nuclei in the muscle was calculated by dividing the total number of nuclei by V.

Determination of DNA. In some of the pigs the indicator muscle from the right fore-foot was removed almost immediately after the death of the animal, cleaned of connective tissue as far as possible and then weighed and stored at $-20°$ until used for the determination of DNA.

DNA was extracted by the method of Zamenhof, Bursztyn, Rich & Zamenhof (1964) and estimated as described by Burton (1956).

RESULTS

Size and number of muscle fibres

Table 1 gives the body-weights, the volumes of the muscles, and the number and diameter of the muscle fibres. The differences between the body-weights of the animals that had received the various treatments have already been described (see p. 422) and these differences in weights are reflected in the diameter of the muscle fibre. It was found, not surprisingly, that the correlation between muscle volume and body-weight (r 0·995) was significantly higher ($P < 0·05$) than the correlation between fibre diameter and body-weight (r 0·937). Mean distributions of fibre diameter for all these groups are shown in Fig. 1; all are unimodal with nearly normal distributions.

For each of the six groups shown in Table 1 a separate one-way analysis of variance

Table 1. *Body-weights, volumes of m. flexor digiti V brevis muscles, and number and diameter of muscle fibres from well-nourished, energy-deficient and protein-deficient pigs*

Nutritional status*	Age	Pig no.	Sex	Body-wt (kg)	Volume of muscle (mm†)	Total no. of fibres	Mean diameter of fibres (μm)
Well-nourished	10 d	F1	♂	3·75	11·7	5420	13·4
		F2	♂	4·65	17·0	5710	15·9
		G1	♀	3·10	10·5	5600	13·4
		G2	♀	3·40	12·0	5890	15·0
		G3	♂	2·93	11·8	6000	16·4
Well-nourished	1 year	A1	♀	215	—	4541	58·0
		B1	♀	225	—	4893	70·1
		C1	♀	206	472	6410	59·5
		D1	♀	208	482	5740	58·3
		E1	♀	167	414	5380	62·2
Energy-deficient	1 year	A2	♂	5·05	—	4571	19·8
		A3	♂	5·75	—	5267	22·3
		C2	♂	7·35	16·9	5864	17·9
		C3	♂	6·30	20·2	6480	19·2
		D2	♂	6·55	24·1	5344	23·1
		D3	♀	6·15	15·7	5680	19·4
Energy-deficient	2 years	A5	♀	11·50	52·1	5305	24·5
		A6	♂	9·65	23·8	4922	24·8
		B3	♀	11·35	24·4	4815	22·3
		B4	♂	10·10	29·4	5052	25·4
		C5	♂	9·65	42·2	6836	25·2
		C6	♀	10·15	39·2	6394	29·1
		C8	♀	10·00	36·2	6526	26·3
Protein-deficient	1 year	A4	♀	15·10	—	4122	31·0
		B2	CM	16·05	—	4942	22·7
		C4	CM	19·40	74·2	5944	30·2
		D4	CM	14·40	28·2	5200	25·1
		E2	CM	13·90	21·1	4860	20·1
Protein-deficient	2 years	A7	CM	27·45	43·0	4355	31·9
		B5	♀	16·90	45·4	4808	29·3
		C7	♀	29·35	91·5	6862	37·6
		D5	♀	29·90	44·0	6885	37·1
		E3	♀	22·55	43·0	5271	31·7

A, B, C, D, E, F, G, litters from which pigs came; CM, castrated male.
* For details, see p. 422.
† For details of calculation, see p. 423.

was made for total number of muscle fibres. Since sex has been found to have no effect on fibre number (Staun, 1963; Rowe, 1968; Stickland, 1973), all animals in the group have been taken together. It was found that, except for the well-nourished, 10-d-old pigs, 'between-animals' variance was very much smaller than 'between-litters' variance. 'Between-animals' variance did not appear to vary in any systematic manner with treatment or age, whereas 'between-litters' variance appeared to increase with age of the animal.

For the next stage of the analysis the well-nourished, 10-d-old animals were omitted because the litters used in this group were different from other groups. For the remaining five groups or treatments a table was produced (Table 2) of the mean

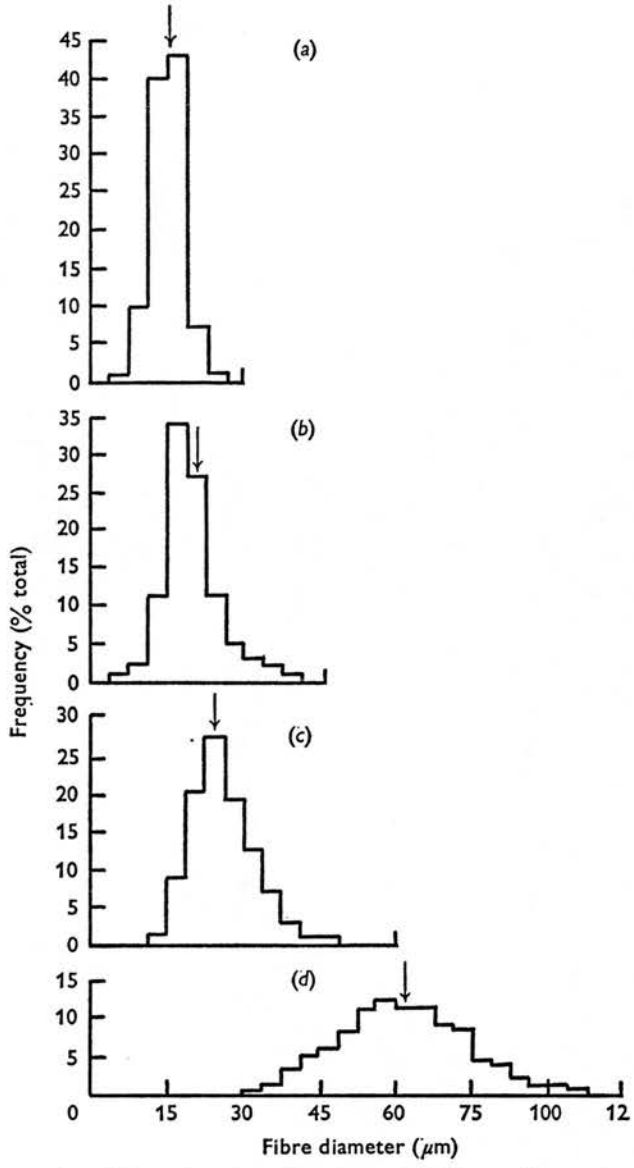

Fig. 1. Distribution of fibre diameters of m. flexor digiti V brevis muscles of pigs. (*a*) Well-nourished, 10-d-old; (*b*) energy-deficient, 1-year-old; (*c*) protein-deficient, 1-year-old; (*d*) well-nourished, 1-year-old. For details of treatments, see p. 422.

observed total fibre number for each treatment from each litter. Mean values could be used because 'between-animal' variation is small relative to 'between-litter' variation. The table was analysed by analysis of variance weighted inversely by the square of the animals' ages in order to correct for the relationship found between age and 'between-litter' variance. The analysis indicated that 'litter-to-litter' variability accounts for most of the variation in fibre number and is highly significant (F 23·57, f_1 4, f_2 12, $P < 0{\cdot}001$). Treatment differences were not significant (F 2·78, f_1 4, f_2 12).

Table 2. *Two-way analysis of variance for total fibre number for m. flexor digiti V brevis muscles from well-nourished, energy-deficient and protein-deficient pigs*

Nutritional status* ...	Well-nourished	Energy-deficient		Protein-deficient	
Age (years) ...	1	1	2	1	2
Litter					
A	4541	4919	5114	4122	4355
B	4893	—	4934	4942	4808
C	6410	6172	6585	5944	6862
D	5740	5512	—	5200	6885
E	5380	—	—	4860	5271
Weighting	1	1	0·25	1	0·25

* For details, see p. 422.

Table 3. *Number of nuclei and amount of DNA in m. flexor digiti V brevis muscles from well-nourished, energy-deficient and protein-deficient pigs*

				Nuclei		DNA	
Nutritional status*	Age	Pig no.	Sex	No./mm³ (× 10³)	Total no. (× 10³)	mg/g muscle	Total (mg/ muscle)
Well-nourished	10 d	F1	♂	388	4530	3·64	0·17
		F2	♂	283	4804	3·57	0·25
		G1	♀	380	3990	3·09	0·13
		G2	♀	395	4740	3·50	0·16
		G3	♂	401	4731	3·22	0·17
Well-nourished	1 year	C1	♀	78·6	37 114	0·84	1·40
		D1	♀	79·0	38 102	0·81	1·17
		E1	♀	85·4	35 316	0·60	0·78
Energy-deficient	1 year	C2	♂	211	3566	—	—
		C3	♂	208	4201	2·13	0·25
		D2	♂	210	5056	—	—
		D3	♀	268	4196	1·61	0·12
Energy-deficient	2 years	A5	♀	128	6675	—	—
		A6	♂	113	2689	—	—
		B3	♀	138	3361	—	—
		B4	♂	115	3377	—	—
		C5	♂	126	5322	—	—
		C6	♀	98	3843	—	—
		C8	♀	114	4123	—	—
Protein-deficient	1 year	C4	CM	118	8759	—	—
		D4	CM	173	4874	1·03	0·14
		E2	CM	211	4462	1·43	0·34
Protein-deficient	2 years	A7	CM	105	4516	—	—
		B5	♀	120	5442	—	—
		C7	♀	107	9789	—	—
		D5	♀	136	5979	—	—
		E3	♀	105	4516	—	—

A, B, C, D, E, F, G, litters from which the pigs came; CM, castrated male.
* For details, see p. 422.

Number of nuclei and amount of DNA

The number of nuclei and amount of DNA in the muscle are shown in Table 3. The number of nuclei/mm³ and amount of DNA (mg/g muscle) was inversely related to body-weight (for number of nuclei per mm³: r 0·424, $P < 0·05$). The values were

highest in the 10-d-old pigs, followed by 1-year-old, energy-deficient, 1-year-old, protein-deficient and finally 1-year-old, well-nourished pigs, in which concentrations of nuclei were relatively low. The total numbers of nuclei were directly related to body-weight (r 0·988, $P < 0·001$) and to muscle weight, i.e., the 1-year-old, control pigs had eight to ten times as many nuclei in their muscles as the animals in the other groups.

DISCUSSION

The results of this study suggest that severe undernutrition of pigs from 10 d of age does not reduce the total number of muscle fibres but it does affect their ability to increase in size. The number of fibres seems to have reached its adult value by 10 d after birth, and undernutrition even as severe as that imposed in this study caused no significant loss of fibres. The genetic make-up of the pig (the litter to which it belongs) on the other hand, is far more important in determining the number of muscle fibres than is the nutritional treatment to which the animal is subjected from 10 d after birth. Whether severe undernutrition before this time of life would affect the number of muscle fibres still remains to be studied.

The present findings are consistent with the theories put forward by other workers, including Luff & Goldspink (1967) working with mice and Staun (1963) working with pigs.

Fibre diameter appears to be related to the body-weight of the animal. Hence, the 1-year-old, severely undernourished pig (mean body-weight 6·19 kg) had smaller fibres than 1-year-old pigs that had been given a diet low in protein (mean body-weight 15·8 kg), and both had smaller fibres than well-nourished, normal pigs of the same age (Table 1 and Fig. 1). Periods of deprivation up to 2 years resulted in a slight increase in muscle fibre diameter and a small increase in body-weight. Differences in body-weight were relatively larger than differences in fibre diameter, but there were also differences in the lengths of the muscles. Volume and weight of the muscles were more closely related to body-weight than were the diameters of the fibres or the lengths of the muscles.

The extent of undernutrition used in the present study was much more severe than any used previously in studies on pigs and sheep, but the results for the number and diameter of muscle fibres are in agreement with other findings for farm animals less severely deprived. Montgomery, Dickerson & McCance (1964) studied the effects of severe undernutrition (comparable to the levels used here) on the sartorius muscle in fowls. They reported that undernutrition prevented the normal increase in number of fibres which takes place in the sartorius muscle of the fowl after it has hatched. Smith (1963), on the other hand, found no increase in the number of muscle fibres in the sartorius muscle of fowls after hatching, and the reason for the failure of the number of fibres to increase, reported by Montgomery *et al.* (1964), is uncertain. Montgomery *et al.* (1964) found, however, that undernutrition of the adult bird did not significantly reduce the total number of muscle fibres.

The histological appearance (transverse sections) of the muscles of the energy-deficient pigs showed that there was much more extracellular space in them than in muscles from well-nourished animals. This is in keeping with the results of Widdow-

son, Dickerson & McCance (1960), who showed that muscles from undernourished pigs contained much more extracellular water than muscles from well-nourished pigs of the same age, and more even than muscles from newborn pigs. The muscles from the energy-deficient pigs contained many small, irregularly shaped fibres as well as some larger, regularly shaped, polygonal ones. Fibres from well-nourished and protein-deficient animals were regularly shaped. From the histogram in Fig. 1 it can be seen that most of the fibres in the muscles of energy-deficient pigs were the same size as those of the 10-d-old animals; these were the irregularly shaped fibres. Some fibres were larger and these cause the mean value for muscles of energy-deficient animals to be higher than those from the 10-d-old animals. All the histograms are unimodal with peaks corresponding approximately to the mean value (Fig. 1) for that particular group. The variation in size of fibres increased as the mean fibre size increased, except for the muscles of the protein- and energy-deficient pigs, which had different mean values but the same distribution width.

In this study the total number of nuclei in the indicator muscle was related to the body-weight and size of the muscle, and so was higher in the heavier pigs. This is in agreement with Montgomery (1962) and Cheek (1968), who found that the number of nuclei increased with muscle growth in man. Concentration of nuclei, however, was highest in pigs with a low body-weight. This is in accordance with the findings of Montgomery *et al.* (1964) for the number of nuclei in the sartorius muscle of fowls.

The results taken as a whole strongly suggest that severe undernutrition, although affecting the size of the fibres in a pig's muscle, does not interfere with their number. The latter seems to be determined by about the time of birth.

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REFERENCES

Abercrombie, M. (1946). *Anat. Rec.* **94**, 239.
Burton, K. (1956). *Biochem. J.* **62**, 315.
Cheek, D. B. (1968). *Human Growth.* Philadelphia: Lea & Febiger.
Goldspink, G. (1964). *J. cell. comp. Physiol.* **63**, 209.
Johnson, E. R. (1971). The growth and development of muscle and fat in the bovine carcase. PhD Thesis, University of Queensland.
Joubert, D. M. (1956). *J. agric. Sci., Camb.* **47**, 59.
Luff, A. R. & Goldspink, G. (1967). *Life Sci.* **6**, 1821.
McCance, R. A. (1960). *Br. J. Nutr.* **14**, 59.
McCance, R. A. (1968). In *Calorie Deficiencies and Protein Deficiencies*, p. 319 [R. A. McCance and E. M. Widdowson, editors]. London: J. & A. Churchill.
McMeekan, C. P. (1940). *J. agric. Sci., Camb.* **30**, 276.
McMeekan, C. P. (1941). *J. agric. Sci., Camb.* **31**, 1.
Montgomery, R. D. (1962). *Nature, Lond.* **195**, 194.
Montgomery, R. D., Dickerson, J. W. T. & McCance, R. A. (1964). *Br. J. Nutr.* **18**, 587.
Rowe, R. W. D. (1968). *J. exp. Zool.* **169**, 59.
Smith, J. H. (1963). *Poult. Sci.* **42**, 283.
Staun, H. (1963). *Acta Agric. scand.* **13**, 293.
Stickland, N. C. (1973). The growth and development of skeletal muscle in pigs. PhD Thesis, University of Hull.
Stickland, N. C. & Goldspink, G. (1973). *Anim. Prod.* **16**, 135.
Strobykina, R. V. (1970). *Svinarstvo, Kyyiv.* **11**, 65.
Widdowson, E. M., Dickerson, J. W. T. & McCance, R. A. (1960). *Br. J. Nutr.* **14**, 457.
Zamenhof, S., Bursztyn, H., Rich, K. & Zamenhof, P. J. (1964). *J. Neurochem.* **11**, 505.

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A DETAILED ANALYSIS OF THE EFFECTS OF VARIOUS FIXATIVES ON ANIMAL TISSUE WITH PARTICULAR REFERENCE TO MUSCLE TISSUE

N. C. STICKLAND, *Department of Veterinary Anatomy and Histology, University of Nairobi, P.O. Box 30197, Nairobi, Kenya*

ABSTRACT. **Nine different fixatives (Carnoy's, Susa, Baker's formalin, 5% formalin, 10% formalin, 10% formol saline, Bouin, Zenker, and 2.5% glutaraldehyde) were compared by two methods. Gelatin-albumin gels were used to study volume changes after fixation and after various stages of subsequent processing. The appearance and hardness of the gels were also noted. The fixatives either shrunk or swelled the gels, but dehydration and clearing shrunk the gels in all cases. Samples of muscle tissue from one location in beef longissimus dorsi muscle were also placed in the different fixatives and processed. Various features were noted for each fixative, including the ease with which the paraffin wax blocks were cut and the staining ability of the sections in Mallory's triple stain. The diameters of the muscle fibers were measured from transverse sections of these samples and compared with the mean diameter of muscle fibers in a frozen unfixed section of muscle tissue. It was found that the fixatives had the same shrinkage effects on both the gels and the muscle samples. Analysis of variance tests showed that the various fixatives caused different degrees of shrinkage. Statistical details are given for the amounts of shrinkage caused by each fixative. Both the general histological picture and the amount of shrinkage were considered when deciding the best fixative. Carnoy was found to be the best of the fixatives investigated.**

The choice of fixative for a particular histological study is often a difficult task owing to the surprising lack of any detailed or statistically valid information on the effects of fixatives on animal tissue. Early work by Baker (1958) concentrated on primary fixatives and included little or no statistical information. The later work of Goldspink (1961), Naudé and Hegarty (1970), and Goldspink *et al.* (1973) demonstrated the effects of some fixatives on the diameter of muscle fibers, but there was little useful statistical information. They all agreed, however, that Flemming's without acetic acid (FWA) is the best fixative for muscle from the shrinkage point of view, but due to the osmium tetroxide in FWA this fixative would be far too expensive for routine study of large muscle samples from meat animals.

As the muscle fiber is the unit structure of skeletal muscle its morphology has been greatly studied by meat scientists. Muscle fiber cross-sectional area or diameter is often used to study various effects, at the cellular level, on the meat of agricultural animals, *e.g.,* levels of nutrition (McMeekan 1940–41, Berry and Kroenig 1967), exercise (Skjervold *et al.* 1963) and breeding experiments (Allen *et al.* 1966) are all studied at the cellular level. The use of muscle fiber size in relation to the lean meat content of animals has also been investigated (Livingston *et al.* 1966). Although muscle fiber size is often used in a comparative manner within an experiment, the results from two experimenters using different fixatives may need to be compared. In order to do this the exact shrinkage caused by each fixative would need to be known.

Owing to the lack of information available, it was felt that a comprehensive study of fixation was required in which the effects of several fixatives on subsequent tissue processing (dehydration, etc.) are investigated. Changes in volume, appearance, and hardness of the tissue should be studied in some detail. Particular reference was made

to fixatives used most widely by meat scientists and to the effect of the fixative on final muscle fiber cross-sectional diameter and on the general histological picture.

Materials and Methods

Histology. The nine fixatives used in this investigation are listed below. Each fixative solution was made up according to the reference quoted. The details for the first three fixatives are also given in Humason (1972).

1. Carnoy, without chloroform (Gatenby and Beams 1950).
2. Susa (Romeis 1948).
3. Formalin (Baker 1958).
4. 5% formalin.
5. 10% formalin.
6. 10% formol saline.
7. Bouin (Humason 1972).
8. 2.5% glutaraldehyde in phosphate buffer (Stickland and Goldspink 1973).
9. Zenker (Humason 1972).

Specimens were fixed for 24 hr except those in Carnoy, which was fixed for 5 hr. Those in glutaraldehyde were fixed at 4 C (Stickland 1973). These fixation times are in accordance with the times recommended by Humason (1972). All specimens were then washed in running water for 48 hr, except those in Carnoy and Susa, which were placed in 70% alcohol for 48 hr, and those in Bouin, which were placed in 50% alcohol for 48 hr. After washing, the specimens were all dehydrated in 70% alcohol for 24 hr (those in Carnoy and Susa went straight to 95% alcohol), then in 95% alcohol for 2 hr followed by two changes of absolute alcohol for 1 hr. The specimens were cleared for 24 hr in cedarwood oil, which was used in preference to xylene as it does not cause hardening of the tissue. This is helpful when fairly large specimens have to be sectioned. Clearing of the muscle samples was followed by infiltration at 58 C in two changes of Paraplast (manufactured by Sherwood Medical Industries Ltd.) for a total of 8 hr. The muscles were then embedded in Paraplast and sectioned at 7 μm on a Leitz rotary microtome. The sections were stained in Mallory's triple stain and mounted in DPX.

Muscles. The muscle samples used for this investigation were all taken from local beef cattle (KMC, Athi River Abbattoir) about 6 hr post-mortem, so that the muscle was in a postrigor condition and did not, therefore, require fixation *in situ*. The samples were all taken from the left longissimus dorsi muscle just below the dorsolateral surface at the thoraco-lumbar junction. The incisions were made at an oblique angle to the axis of the muscle so that the samples were orientated in the direction of the fibers of the longissimus dorsi muscle (as explained by Eisenhut *et al.* 1966). Hence, when the samples were sectioned on the microtome the muscle fibers were sectioned as transversely as possible. Care was also taken to remove the samples (each with a volume of about 1 cc) from as small an area as possible so that errors due to "inter-location" and "inter-position" fiber size differences were eliminated (Swanson *et al.* 1965). Ten muscle samples were removed from each animal. One of these samples was frozen quickly using liquid Freon (−160 C) and stored in liquid nitrogen for the duration of the journey back to the laboratory where it was

immediately sectioned at 10 μm using a Bright Co. cryostat. The sections were picked up on clean glass slides, stained with methylene blue, and mounted in glycerine jelly. The other nine muscle samples were fixed in the nine different fixatives listed above. Further processing of these samples was then carried out as previously described. The muscle sections were photographed using a Leitz microscope camera (automatic exposure).

Fiber diameter measurements. These measurements were carried out on the muscle sections. The method used was that employed by Song *et al.* (1963) whereby two orthogonal diameters of the muscle fiber were measured. The polygonal form of the muscle fiber can be inscribed within an ellipse, the area of this form being proportional to its major and minor axes. Similar measurements of the two axes were advocated by Aherne (1970) for determining the cross-sectional area of muscle fibers. In this investigation the mean of the two axes was taken as the diameter of the muscle fiber being measured. Measurements were made on consecutive fibers by traversing the histological sections horizontally and vertically with a graduated mechanical stage to ensure uniform sampling. An ocular micrometer scale was used to measure 100 muscle fibers for each section of muscle. This number was calculated statistically by Meara (1947) to give a reliable mean fiber size measurement.

Gelatin-albumin gels. It was decided that the best way of studying volume changes after fixation and after various stages of subsequent processing might be to use gelatin-albumin gels. This gel has been used in experiments on primary fixatives by Baker (1958). It contains globular protein (albumin) and a fibrous protein (gelatin, a derivative of collagen) and so may be used as a crude model of protoplasm. It is made up by dissolving gelatin at 15% in warm diluted egg white (1:2 parts water) (Baker 1958). The refractive index of the resultant gel is about 1.365, which is within the same range shown by protoplasm (1.353). This will mean that its protein content of 19.4% is also about the same. The gel was allowed to set in a large flat mold and then cut into cubes about 1 cc in volume. The gels were processed in the various fixatives, washed, and dehydrated as described above. The volumes of the cubes were noted after fixation, after washing, after 70% alcohol, after absolute alcohol, and after clearing in cedarwood oil. The gels were not processed beyond clearing. The volumes of the gel cubes were measured by displacement of fluid in a narrow graduated tube. The fluid used was the one from which the cube had been removed during processing. The appearance and hardness of the gels were also noted at different stages.

Statistical methods. The first test carried out on the results was a correlation analysis between log (final volume of gel) and log (mean fiber diameter of muscle fibers). The results were paired for each fixative solution so that there were nine pairs of results. The analysis was carried out by the method of Bartlett (1949) in that both variables were subject to error. The correlation analysis would show whether the effects of the various fixatives was the same on both the muscles and the gels.

An analysis of variance test was carried out on the fiber diameter results for each fixative to test whether the fixatives had any effect on the measurements. The results for each fixative were compared to establish real differences between them by the method of calculating the least significant difference (Snedecor and Cochran 1967). Similar analyses were carried out on the final gel volumes.

Results

The correlation analysis between log (gel volume) and log (fiber diameter) gave a correlation coefficient of 0.9746, which was highly significant ($P < 0.001$). The figures used for this correlation were the last columns of figures given in Tables 1 and 2. Log values were used in order to investigate the linear relationship between gel volume and fiber diameter.

Shrinkage of muscle fibers. The mean muscle fiber diameter measurements on the frozen unfixed sections of the four animals were 52.9, 56.9, 49.5, and 54.8 μm. It can be seen, therefore, that the variation between animals is fairly small, but for more exacting comparative measurements the diameters of the fixed muscle fibers were expressed as a percentage of the frozen, unfixed fibers. These muscle fiber measurements are shown in Table 1. An analysis of variance on these figures gives a highly significant F ratio (Table 1). Individual fixative differences were investigated by calculating the least significant difference (LSD) (Snedecor and Cochran 1967) which was 3.00 at the 5% level for the results of Table 1. Hence, the difference between a specific pair of means is significant at the 5% level if it exceeds this figure. The significance values shown in Table 3 were calculated in this manner. It may be pointed out here that the 95% confidence limits for the population difference between any pair of means are given by adding ± 3.00 to the observed difference, *e.g.*, there is a 95% certainty that the muscle fiber diameter shrinkage caused by Susa will be between 7.80 and 13.80% less than that caused by Zenker.

Appearance and other notable features of the muscle sections. Several features of the muscle tissue were noted, including the hardness of the tissue while sectioning. It was found that glutaraldehyde was the hardest to cut, followed by Carnoy, Susa, 10% formol saline, and 10% formalin. Muscles fixed in 5% formalin, Baker's formalin, Bouin, and Zenker were easiest to cut. Except for glutaraldehyde, however, the differences were only slight. It was found that the Susa-fixed sections tended to overstretch on the hotplate in comparison with other sections. The degree of staining in Mallory's triple stain was strongest in Carnoy, Susa, glutaraldehyde, and Zenker sections, followed by Bouin sections, then 10% formol saline, 5% formalin, 10% formalin, and last, Baker's formalin, which stained very poorly. Photomicrographs of the

Table 1. Effects of Different Fixatives on the Diameter of Muscle Fibers (Given as a Percentage of the Mean Diameter of Frozen, Unfixed Muscle Fibers)

Fixative	Individual Results				Mean
	1	2	3	4	
Carnoy	75.5	75.2	72.0	73.0	73.9
Susa	74.3	74.5	71.2	71.0	72.8
Baker's formalin	85.2	82.5	80.0	83.0	82.7
5% formalin	68.4	69.8	72.8	66.2	69.3
10% formalin	69.2	70.1	68.6	68.2	69.0
10% formol saline	71.9	68.7	67.6	70.8	69.8
Bouin	78.3	74.6	79.7	75.1	76.9
Glutaraldehyde	70.8	70.6	73.1	68.2	70.7
Zenker	58.9	63.6	61.6	63.7	62.0
				Analysis of variance:	F ratio = 31.20 $P < 0.001$

TABLE 2. EFFECTS OF DIFFERENT FIXATIVES ON THE VOLUME OF GELATIN-ALBUMIN CELLS AFTER VARIOUS STAGES OF PROCESSING (GIVEN AS PERCENTAGES OF THE INITIAL VOLUME)

All figures are a mean of four except "After cedarwood oil" where individual figures as well as means are given. Any deviation from the processing schedule, given by the column headings, is noted.

Fixative	After 5 hr in fixative	After 24 hr in fixative	After 48 hr in water	After 24 hr in 70% alc.	After 95% & Abs. alc.	After cedarwood oil				
						1	2	3	4	Mean
Carnoy	90	—	186[1]	167[2]	108	39	34	36	38	36.8
Susa	65	50	54[1]	55[2]	48	34	34	33	35	34.0
Baker's formalin	114	118	122	76	58	41	45	48	44	44.5
5% formalin	131	132	151	80	56	33	34	33	33	33.3
10% formalin	109	106	114	71	50	33	34	34	33	33.5
10% formol saline	116	119	136	92	55	34	32	32	34	33.0
Bouin	89	80	79[3]	96	63	44	43	44	43	43.5
Glutaraldehyde	86	95	104	73	56	35	37	36	36	36.0
Zenker	90	86	97	61	50	23	25	24	23	23.8

Analysis of variance: F ratio = 76.63, $P < 0.001$

[1] After 24 hr in 70% alcohol.
[2] After a further 48 hr in 70% alcohol.
[3] After 48 hr in 50% alcohol.

TABLE 3. SIGNIFICANT DIFFERENCES BETWEEN INDIVIDUAL FIXATIVES (BY LEAST SIGNIFICANT DIFFERENCE AT $P < 0.05$)

Comparisons between muscle fiber diameters are shown on the left of each column; comparisons between gel volumes on the right.

	Susa		Baker's formalin		5% formalin		10% formalin		10% formol saline		Bouin		Glutaraldehyde		Zenker	
Carnoy	NS	*	*	*	*	*	*	*	*	*	NS	*	*	NS	*	*
Susa			*	*	*	NS	*	NS	NS	NS	*	*	NS	NS	*	*
Baker's formalin					*	*	*	*	*	*	*	NS	*	*	*	*
5% formalin							NS	NS	NS	NS	*	*	NS	*	*	*
10% formol saline									NS	NS	*	*	NS	*	*	*
Bouin											*	*	NS	*	*	*
Glutaraldehyde															*	*

NS, not significant; *, significant.

frozen and fixed sections are shown in Figure 1. The histological picture of the muscle sections was assessed by the ease with which muscle fibers could be distinguished for measurements to be taken easily, *e.g.*, diameter and number of muscle fibers. It was found that the order from best to worst was glutaraldehyde, Carnoy, 10% formol saline, Zenker, Baker's formalin, Susa, 10% formalin, 5% formalin, and last, Bouin sections in which the fibers were very indistinct. It should be noted, however, that some of the poorer fixatives, regarding the above criterion, were good for intracellular components, *i.e.*, myofibrils It can be seen from Figure 1 that the myofibrils were especially distinct in Carnoy sections and fairly distinct in Susa, Baker's formalin, 10% formol saline, and Bouin-fixed sections.

Shrinkage of gels. Table 2 shows the volumes of the gels, as percentages of the initial volumes, after fixation and after various stages of subsequent processing. Carnoy, Susa, Bouin, Zenker, and glutaraldehyde seem to cause some amount of shrinkage, whereas all the formalin-based fixatives seem to cause some swelling of the

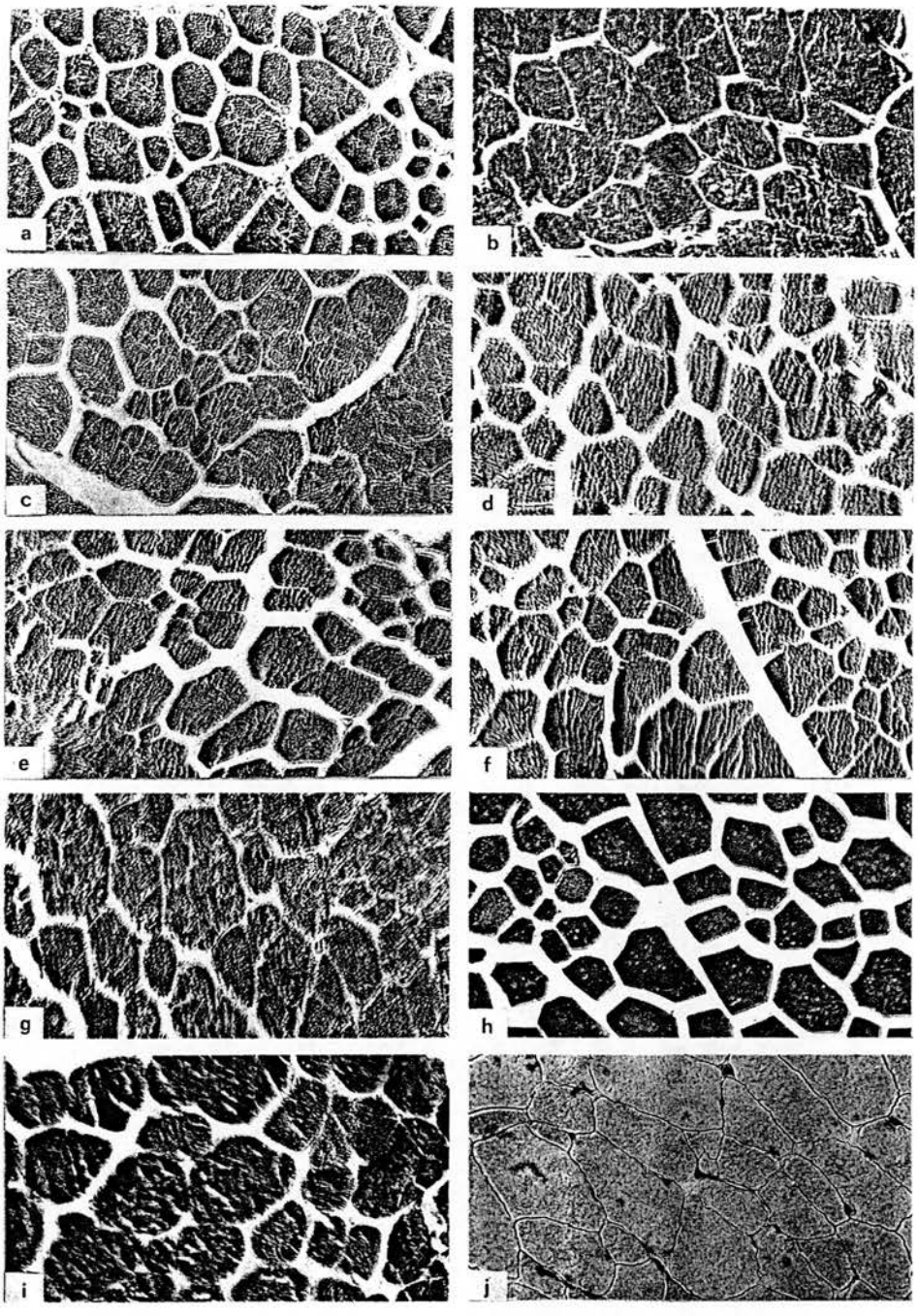

FIG. 1. Photomicrographs of transverse sections of muscle tissue fixed in the following solutions: a: Carnoy; b: Susa; c: Baker's formalin; d: 5% formalin; e: 10% formalin; f: 10% formol saline; g: Bouin; h: glutaraldehyde; and i: Zenker. j is a cryostat section of fresh, unfixed muscle. × 350.

gels. It was found that gels in water swelled tremendously (290% in 18 hr and 365% in 68 hr). Subsequent washing in water causes some slight swelling of most of the gels. Carnoy-fixed gels washed in 70% alcohol appeared to swell quite considerably, whereas Susa and Bouin-fixed gels washed in 70% alcohol and 50% alcohol, respectively, showed little change in volume. Subsequent dehydration in 70% alcohol, 95% alcohol, and absolute alcohol caused shrinkage of all the gels at each stage, except anomalously for Bouin-fixed gels which appeared to swell after 70% alcohol. (This was true for all four of the gels). Clearing in cedarwood oil caused more shrinkage in all cases. An analysis of variance was carried out on these final volumes and gave a highly significant F ratio ($P < 0.001$) (see Table 2). Individual fixative differences are shown in Table 3. (The least significant difference for the final values in Table 2 was 2.04 at the 5% level.)

Appearance of gels. The appearance of the gels and their hardness were noted after complete dehydration. The degrees of hardness used were hard, very firm, firm, quite firm, soft, and very soft. It was found that Carnoy gels were very soft; Bouin gels were quite firm; Susa, Baker's formalin, 5% formalin, and Zenker gels were firm; 10% formalin and 10% formol saline gels were very firm, and glutaraldehyde gels were very firm to hard. All gels had much the same hardness after cedarwood oil. After dehydration the Carnoy gels appeared cloudy; Susa gels appeared very white and opaque; Baker's formalin, 5% formalin, 10% formalin, and 10% formol saline gels were clear with no color change; glutaraldehyde gels were also clear but more yellow in color; Bouin gels were yellow and opaque, and Zenker gels were brown and opaque.

Discussion

The highly significant correlation between log (gel volume) and log (fiber diameter) demonstrates that it may be reasonably assumed that the effect of each fixative was the same on both the gelatin-albumin gels and the muscle fibers as far as shrinkage was concerned. A shrinkage in gel volume to 33.0% (as for 10% formol saline-fixed gels) would mean a shrinkage in one linear dimension (assuming shrinkage is the same in all directions) to 69.0% of the original. The actual muscle fiber diameter shrinkage of 10% formol saline-fixed muscles was to 69.8%, which is in very close agreement. All the fixatives show a similar trend. Hence, an assumption that the gel volume shrinkage represents muscle volume shrinkage appears to be quite valid. We can, therefore, probably say that the volume changes at the various stages shown in Table 2 will be similar for the muscle tissue and probably for other animal tissue. It would seem, therefore, that Baker's (1958) gelatin-albumin gels are indeed a good model of protoplasm for investigating fixative effects.

An important point to be noticed from Table 2 is that the volume changes caused by the fixatives alone bear little or no relationship to the final volumes after subsequent dehydration and clearing. This point was noted by Hertwig (1931) who found that mercuric chloride (saturated aqueous) and 4% formaldehyde caused swelling of unfertilized eggs of *Arbacia pustulosa* whereas ethanol caused shrinkage. Subsequent dehydration and clearing caused shrinkage in all cases. A lot of work on fixation has missed this point or at least has not mentioned it. It can be seen from Table 2 that the four formalin fixatives cause swelling of the tissue which is in agreement with Hertwig

(1931), whereas the four fixative mixtures cause shrinkage. Subsequent dehydration in ethanol and clearing in cedarwood oil caused shrinkage at each stage. The amount of this shrinkage must depend on the ability of the initial fixative to prevent this. The analysis of variance in Table 2 proves that this ability varies from one fixative to the next. Very little data is available on the final volume shrinkage caused by fixatives although the shrinkage to 33% caused by 5% formalin (Table 2) is in very close agreement with the shrinkage of the primary spermatocytes of *Helix aspersa* to 34% fixed in the same way (Ross 1953). It should be mentioned here that infiltration and embedding in Paraplast wax may presumably cause some small amount of shrinkage due mainly to the shrinkage effects of heat on the collagen. Low melting point wax would, therefore, be preferable (Naudé and Hegarty 1970).

The different opacities mentioned above are due to coagulant fixatives (Baker 1958) being present in the fixative mixtures. Formalin and glutaraldehyde are noncoagulants. The hardness of the gels is related to the hardness after fixation, but dehydration and clearing inevitably accentuate this.

Experiments on fixation of muscle have either considered few fixatives or have contained very little statistically significant information. Naudé and Hegarty (1970) and Goldspink (1961) showed that FWA shrunk muscle tissue much less than formalin. As already mentioned, FWA was not considered here owing to its expense when using large muscle from agricultural animals. Goldspink (1961) showed that Carnoy, Bouin's, and neutral formalin caused approximately a 45% shrinkage of muscle fiber area. Thus, the fiber diameter would be reduced to about 70% of the fresh tissue (assuming the fibers to be cylindrical). This is in approximate agreement with the results given here. No statistical evidence was given for a difference between these fixatives. Later work by Goldspink *et al.* (1973) did show, however, that there was no significant difference between fixation effects of Carnoy and glutaraldehyde fixatives on muscle fiber diameter. This is in agreement with the results of Table 1, the statistical analysis being shown in Table 3. Dreyer *et al.* (1972) investigated the effects of Carnoy, 10% formalin, and Zenker fixatives on porcine muscle fibers, but found no significant differences between them (although Carnoy appeared best in one particular case). It should be pointed out, however, that their cryostat sections anomalously provided rather low fiber diameter measurements (only about 7–15% greater than those of fixed sections) in contrast to the results given here and the results of other workers already mentioned.

The results given in Tables 3 and 4, when taken as a whole, suggest that the best fixatives for preventing shrinkage are Baker's formalin, Bouin, and Carnoy, followed by Susa and glutaraldehyde together with, or just above, the remaining formalin fixatives with Zenker being the worst. It must be remembered, however, that glutaraldehyde and Carnoy provided the best histological picture for distinguishing muscle fibers. It was also noted that glutaraldehyde was very hard to cut; Carnoy was somewhat better and also was among the best three regarding shrinkage, whereas glutaraldehyde was not. If both the shrinkage and the histological picture are considered (and also hardness, to a small extent) then Carnoy is probably the best fixative of those investigated here, followed by glutaraldehyde and Baker's formalin, then 10% formol saline and Bouin, followed by Susa, then 10% formalin and Zenker, and, last, 5% formalin.

TABLE 4. RELATIVE SHRINKAGE OF MUSCLE FIBERS AND GELS IN DIFFERENT FIXATIVES

Order of least shrinkage	Fiber diameter	Gel volume
1st	Baker's formalin	Baker's formalin; Bouin
2nd	Bouin[1]	Carnoy; glutaraldehyde[4]
3rd	Carnoy[1]; Susa[2, 3]	Susa[4]; 5% formalin; 10% formalin; 10% formol saline
4th	10% formol saline[2]; glutaraldehyde[3]; 5% formalin; 10% formalin	Zenker
5th	Zenker	

[1, 2, 3, 4] Differences between fixatives with same superscripts not significant.

It is apparent from the work described here that fixatives have widely varying effects on muscle tissue. The considerable amount of shrinkage produced by these fixatives is probably not fully realized by many cytologists and histologists. As already mentioned, the muscle fibers of animals are greatly studied by physiologists, meat scientists, muscle histologists, and many others. It may be useful to many workers to know how far measurements on fixed tissue can be related to the unfixed, fresh tissue. The results described here also give some clear indications of the best fixatives to use for the histological study of muscle and other animal tissue.

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REFERENCES

Aherne, W. 1970. Quantitative methods in histology. J. Med. Lab. Technol. *27:* 160–170.

Allen, E., Forrest, J. C., Chapman, A. B., First, N., Brag, R. W., and Briskey, E. J. 1966. Phenotypic and genetic associations between porcine muscle properties. J. Anim. Sci. *25:* 962–966.

Baker, J. R. 1958. *Principles of Biological Microtechnique.* Methuen & Co., London.

Bartlett, M. S. 1949. Fitting a straight line when both variables are subject to error. Biometrics *5:* 207–212.

Berry, B. W. and Kroenig, G. H. 1967. Effect of ration and sample location on porcine muscle fiber diameter and tenderness. J. Anim. Sci. *26:* 892.

Dreyer, J. H., Naudé, R. T., and Gouws, P. J. 1972. The influence of slaughter technique and histological treatment on muscle fiber diameter of low and high pH_1 pork muscle. S. Afr. J. Anim. Sci. *2:* 109–112.

Eisenhut, R. C., Cassens, R. G., Bray, R. W., and Briskey, E. J. 1966. Fiber arrangement and microstructure of bovine longissimus dorsi muscle. J. Food Sci. *30:* 955–959.

Gatenby, J. B. and Beams, H. W. 1950. *The Microtomist's Vade-Mecum.* J. and A. Churchill, London.

Goldspink, G. 1961. Fixation of muscle. Nature *192:* 1305.

Goldspink, G., Gelder, S, Clapison, L., and Overfield, P. 1973. Pre- and post-rigor fixation of muscle. J. Anat. *114:* 1–6.

Hertwig, G. 1931. Der Einfluss der Fixierung auf das Kern-und Zellvolumen. Zeit. mikr.-anat. Forsch. *23:* 484–504.

Humason, G. L. 1972. *Animal Tissue Techniques.* 3rd ed. W. H. Freeman and Company, San Francisco.

Livingston, D. M. S., Blair, R., and English, P. R. 1966. The usefulness of muscle fiber diameter in the studies of lean meat content of pigs. Anim. Prod. *8:* 267–275.

McMeekan, C. P. 1940–41. Growth and development in the pig with special reference to carcass quality characters. I–V. J. Agric. Sci. *30:* 276 and J. Agric. Sci. *31:* 1.

Meara, P. J. 1947. Post-natal growth and development of muscle, as exemplified by the gastrocnemius and psoas muscles of the rabbit. Onderstepoort J. Vet. Sci. *21:* 329–466.

Naudé, R. T. and Hegarty, P. V. J. 1970. The effect of fixation on the size of skeletal muscle fibres embedded in ester wax. Proc. S. Afr. Soc. Anim. Prod. *9:* 217–220.

Romeis, B. 1948. *Mikroskopische Technik*. Leibniz Verlag, Munich.

Ross, K. F. A. 1953. Cell shrinkage caused by fixatives and paraffin-wax embedding in ordinary cytological preparations. Quart. J. Micr. Sci. *94:* 125–139.

Skjervold, H., Standal, N., and Bruflot, R. 1963. Effect of one form of exercise on the body development in pigs. J. Anim. Sci. *22:* 458–462.

Snedecor, G. W. and Cochran, W. G. 1967. *Statistical Methods*, 6th ed. Iowa State University Press, Ames.

Song, S. K., Shimada, N., and Anderson, P. J. 1963. Orthogonal diameters in the analysis of muscle fibre size and form. Nature *200:* 1220–1221.

Stickland, N. C. 1973. The Growth and Development of Skeletal Muscle in Pigs. Ph.D. Thesis. University of Hull, Hull, England.

Stickland, N. C. and Goldspink, G. 1973. A possible indicator muscle for the fiber content and growth characteristics of porcine muscle. Anim. Prod. *16:* 135–146.

Swanson, L. A., Kline, E., and Goll, D. 1965. Variability of muscle fiber size in bovine longissimus dorsi. J. Anim. Sci. *24:* 97–101.

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SHORT NOTE

Number of fibres in the skeletal muscle of miniature pigs

By N. C. STICKLAND* and G. GOLDSPINK
Department of Zoology, The University, Hull

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There is evidence that selection for small body size in mice results in a decrease in the total number of muscle fibres in a given muscle (Luff & Goldspink, 1967; Hanrahan, Hooper & McCarthy, 1973). It has also been shown that small chickens have fewer muscle fibres in certain muscles than larger chickens (Smith, 1963). This type of work has not, however, been extended to a study of the larger domestic animals. At Göttingen in Germany, Haring *et al.* (1966) have developed a breed of miniature pig which, at 6 months of age, was about a third of the body weight of commercial pigs. We therefore decided to investigate the muscles of these miniature pigs and compare them with commercial pig muscles, paying particular attention to the number of muscle fibres.

MATERIALS AND METHODS

In a previous paper (Stickland & Goldspink, 1973) we found that the number of muscle fibres in m. flexor digiti V brevis in pigs was closely correlated with the numbers in other muscles of the same animal. We decided, therefore, to use this 'indicator' muscle in this investigation. For the commercial pigs, trotters from the left fore limb of six Large White pigs were removed soon after slaughter and placed in containers of 2·5% glutaraldehyde in phosphate buffer (Stickland & Goldspink, 1973). These pigs had been reared on a Meat and Livestock Commission Testing Station (Selby, U.K.). For the miniature pigs a similar procedure was carried out at Göttingen in Germany using the pigs developed by Haring *et al.* (1966). All 12 pigs used were of similar ages (about 6–7 months). In the laboratory m. flexor digiti V brevis was dissected out from the trotters. The muscles were embedded in paraplast, sectioned to obtain 7 μm transverse sections through the 'belly' of the muscle and stained with Heidenhain's haematoxylin. This muscle was small enough to permit the counting of the total number of muscle fibres in the muscle cross-section using a Leitz projection microscope. The density of muscle fibres was established by counting the number of fibres in an area (0·75 mm^2), with little connective tissue, outlined by a square eye-piece graticule.

* Present address: Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh, EH9 1QH.

RESULTS

The results of the investigation are shown in Table 1 together with the statistical significance, measured by Student's *t*-test, of the difference between the Large White and miniature pigs for the various parameters. It can be seen from Table 1 that m. flexor digiti V brevis of the Large White pigs had about 68% more muscle fibres than that of the miniature pigs, and the body weight of the Large White pigs was about 3·6 times greater than that of the miniature pigs at a similar age.

DISCUSSION

It would seem that the number of muscle fibres in the 'indicator' muscle of miniature pigs is significantly less than in commercial Large White pigs (Table 1). The number of fibres in the 'indicator' muscle has been shown to be correlated with the number of muscle fibres in other larger muscles of the same animal for various breeds and cross-breeds of pigs (Stickland & Goldspink, 1973). Therefore, it is likely that other muscles in these miniature pigs also have a small number of fibres. As already mentioned, Luff & Goldspink (1967) and Hanrahan *et al.* (1973) found that large mice had more muscle fibres in certain muscles than smaller mice. Smith (1963), working on chickens, also found that larger chickens had more fibres in the sartorius muscle than did smaller chickens. It appears, therefore, that the results presented here agree with work of a similar nature on number of muscle fibres in other animals.

As far as muscle fibre diameter is concerned, the results suggest that there is no significant difference between the Large White and miniature pigs. Table 1 shows no significant difference in muscle fibre density which would be a function of

Table 1. *Body weight and number and density of muscle fibres in m. flexor digiti V brevis in Large White and miniature pigs of similar age*

	Large White	Miniature pigs	Significance of difference
No. of animals	6	6	—
Age (months)	*Ca.*6	6·8 ± 0·8	—
Body weight (kg)	101·3 ± 3·0	28·1 ± 2·9	$P < 0.001$
Muscle fibre density (no./0·75 mm²)	381 ± 45	283 ± 31	$P > 0.05$
Total no. of muscle fibres	5050 ± 352	3011 ± 202	$P < 0.001$

Mean values are given with standard errors.

fibre diameter. This is in apparent contradiction to the results of the authors mentioned above who found that smaller animals had smaller diameter fibres, although this did not appear to be as important as number of fibres in limiting muscle size. It should be mentioned here that, although the number of muscle fibres is only 1·7 times greater in the Large White pigs and body weight is 3·6 times greater, there will also be a difference in muscle fibre length.

The results of this investigation appear to show that the main factors controlling muscle size in pigs are number and length of fibres; we did not establish a significant difference in diameter.

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REFERENCES

HANRAHAN, J. P., HOOPER, A. C. & MCCARTHY, J. C. (1973). Effects of divergent selection for body weight on fibre number and diameter in two mouse muscles. *Animal Production* **16**, 7–16.

HARING, F., GRUHN, R., SMIDT, D. & SCHEVEN, B. (1966). Miniature swine development for laboratory purposes. In *Swine in Biomedical Research* (ed. L. K. Bustad and R. O. McClellan), pp. 789–796. Washington: Battelle Memorial Institute.

LUFF, A. R. & GOLDSPINK, G. (1967). Large and small muscles. *Life Sciences* **6**, 1821–1826.

SMITH, J. H. (1963). Relation of body size to muscle cell size and number in the chicken. *Poultry Science* **42**, 283–290.

STICKLAND, N. C. & GOLDSPINK, G. (1973). A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16**, 135–146.

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Muscle weights and succinic dehydrogenase distribution in the hind limb musculature of two rodents *(Thryonomys gregorianus* and *Pedetes capensis)* with different locomotory habits

N. C. Stickland[1]

Department of Veterinary Anatomy, University of Nairobi, Nairobi, Kenya

Key words. Rodents · Muscles · Muscle fibre types · Succinic dehydrogenase · Locomotion

Abstract. The hind limb muscles of the spring hare *(Pedetes capensis)* were found to be relatively heavier than the hind limb muscles of the cane rat *(Thryonomys gregorianus)*. The distribution of succinic dehydrogenase activity was investigated in four of these muscles (m. gluteus superficialis, m. semimembranosus, m. biceps femoris and m. rectus femoris) from both animals. It was found that the spring hare had a higher proportion of low-activity fibres in all four muscles than the cane rat. All muscle fibre types were also smaller in diameter in the spring hare than the cane rat. These results are discussed in relation to the different locomotory habits of the two animals.

Introduction

The functional significance of the distribution of various muscle fibre types demonstrated histochemically in skeletal muscle is still being elucidated. Muscle fibre types have been studied in various muscles [e.g. *Padykula,* 1952; *Romanul,* 1964; *Edgerton and Simpson,* 1969] but only in the diaphragm [*Gauthier and Padykula,* 1966; *Davies and Gunn,* 1972] has a real attempt been made to explain the difference in muscle fibre type distribution found in the same muscle of different animals. The difference was found to be related to the degree of activity of the diaphragm in the animals studied. It was decided, therefore, to make use of an opportunity to look at some hind limb muscles of two related animals but with different locomotory habits. When disturbed, the cane rat *(Thyronomys gregorianus)* moves off quickly on all four relatively short limbs (fig. 1), whereas the spring hare *(Pedetes capensis)* uses only its relatively longer and more developed hind limbs (fig. 1) to take long jumps, up to 9 m, in order to move away [*Walker,* 1964; personal obs.]. An attempt was therefore made to examine how the hind limb muscles have adapted, if at all, on both a gross and a histochemical level, to this different locomotory behaviour.

[1] The author gratefully acknowledges the technical assistance of Mr. *S. Paye.*

Material and methods

3 cane rats *(Thyronomys gregorianus)* and 3 spring hares *(Pedetes capensis)* of similar known body weights were caught near Nairobi, Kenya, over a period of a few days and killed by inhalation of chloroform.

From the left hind limb of each animal small pieces of muscle were removed from m. gluteus superficialis, m. semimembranosus, m. biceps femoris and

m. rectus femoris. These muscles were chosen because of their different functions and anatomical locations. The muscle samples were always removed from a similar position, usually the anterior border, for each given muscle. The muscle samples were placed on a Leitz freezing microtome and rapidly frozen by spraying with dichlorodifluoromethane (Arcton 12, ICI Ltd). 15-μm sections were cut at −20 °C and stained for succinic dehydrogenase (SDHase) activity by the method of *Nachlas et al.* [1957]. Sections were also stained for fat using Sudan IV. By means of a Leitz projection microscope the number of muscle fibres (red, white or intermediate) per square millimetre was counted in the sections stained for SDHase activity. The average size of muscle fibres was estimated by measuring the diameters of 100 fibres of each type, using a calibrated eyepiece graticule. It should be noted here that the classification of muscle fibres into red, white or intermediate was based on the degree of SDHase activity, red being high and white low.

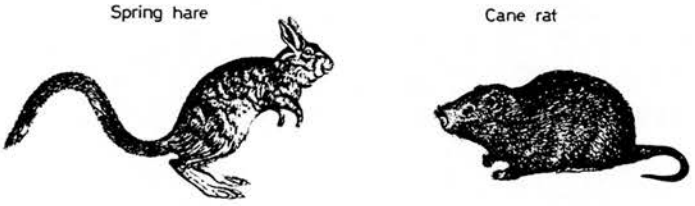


Fig. 1. Drawing of a spring hare and a cane rat to show the difference in hind limb development.

Table I. Muscle weights as a percentage of body weight

Muscle	Function	Cane rat	Spring hare	Spring hare / Cane rat
Gluteus superficialis[1]	flex hip	0.83	1.71	2.1
Tensor fasciae latae	flex hip	0.21	0.32	1.5
Rectus femoris	extend stifle	0.07	0.26	3.7
Vastus lateralis, medialis et intermedius	extend stifle	0.87	1.95	2.2
Biceps femoris[1]	extend hip	1.21	1.82	1.5
Gluteus medius[1]	extend hip	0.60	1.40	2.3
Adductor[2]	extend hip	0.30	0.53	1.8
Semimembranosus[2]	extend hip	0.88	1.40	1.6
Semitendinosus	extend hip	0.16	0.40	2.5
Gastrocnemius et flexor digitorum superficialis	flex stifle	0.88	1.41	1.6
Gracilis[2]	flex stifle	0.19	0.33	1.7

[1] Abduct limb.
[2] Adduct limb.

The right hind limb of each animal was dissected and all the muscles cleared of fat and excess connective tissue. Several muscles (listed in table I) were then removed entire and weighed.

Results

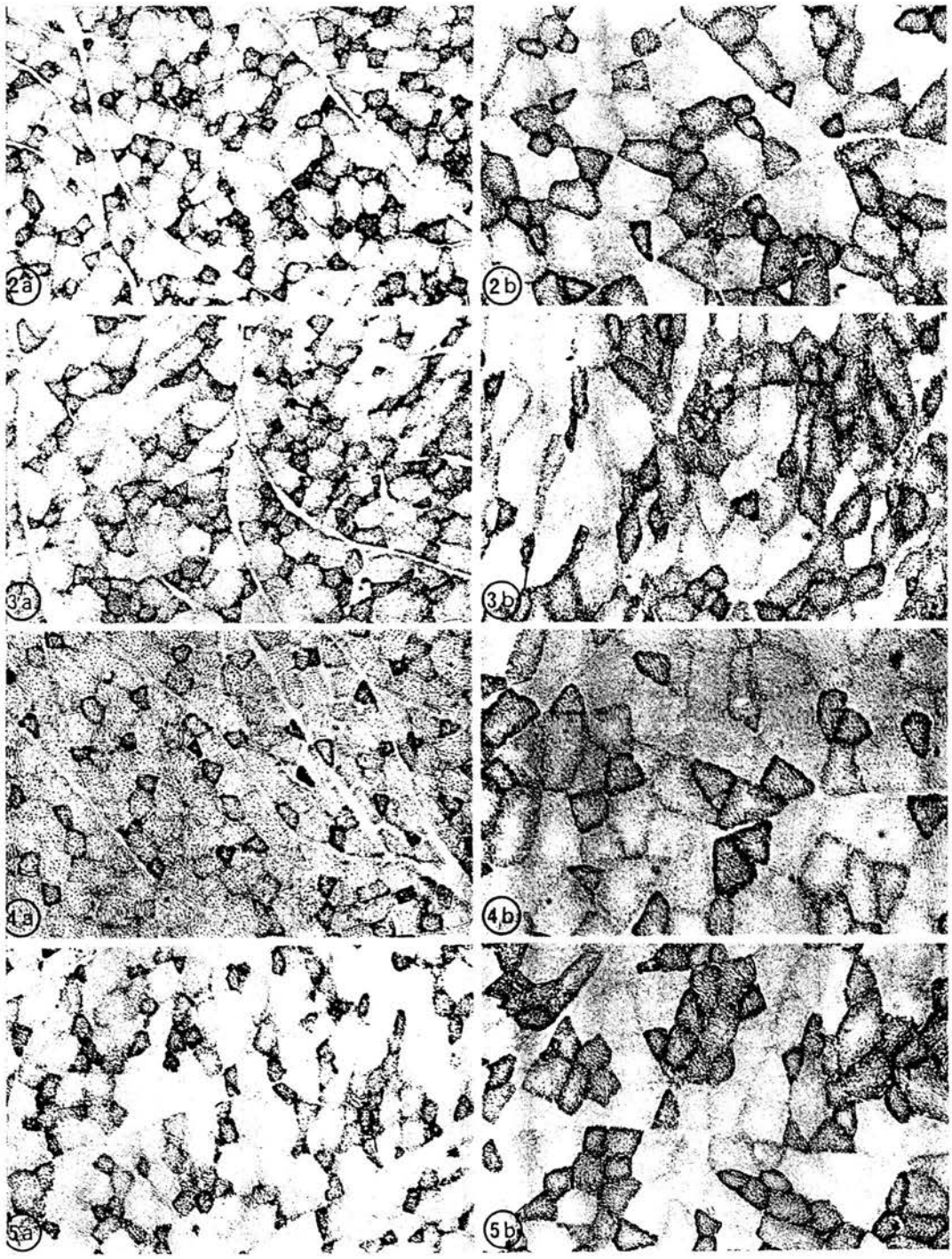
The entire body weights of the 3 cane rats was 2,233 ± 186 g and that of the 3 spring hares was 2,367 ± 176 g. Table I shows the weights of individual hind limb muscles (from one side only) as a percentage of the total body weight. Each figure is an average for the 3 animals of each species. The last column of figures shows that the spring hare muscles were relatively much heavier (the figures show exactly how much) than the cane rat muscles. The function of each muscle is also indicated in table I.

Table II shows the number per square millimetre, percentage (by number) and mean size (diameter) for each type of muscle fibre in four hind limb muscles of both cane rat and spring hare. The figures given are averages for the 3 animals of each species. In each muscle, all muscle fibre types were larger in the cane rat than the spring hare. There also appeared to be a higher percentage of white muscle fibres in the spring hare than the cane rat in all four muscles studied. Figures 2–5 show photomicrographs of the sections used for analysis. The sections stained for fat gave a similar picture with muscle fibres high in fat content corresponding to those fibres high in SDHase activity.

Table II. Analysis of muscle fibre types (based on SDHase activity)

Muscle fibres	Gluteus superficialis		Semimembranosus		Biceps femoris		Rectus femoris	
	CR	SH	CR	SH	CR	SH	CR	SH
Red								
n/mm²	92	141	58	130	68	116	35	51
%	49	30	28	28	28	25	23	13
Size, μm	40.9	27.0	44.4	27.0	46.9	26.9	52.4	25.3
Intermediate								
n/mm²	47	67	46	71	91	145	36	59
%	25	14	22	15	38	32	24	14
Size, μm	56.2	35.8	68.2	42.5	61.6	36.6	68.7	37.2
White								
n/mm²	48	270	105	261	81	195	81	294
%	26	56	50	57	34	43	53	73
Size, μm	76.4	58.1	89.4	63.8	78.5	48.0	84.9	50.7
Total								
n/mm²	187	478	209	462	240	456	152	404
Size, μm	54.0	45.6	72.1	50.3	63.2	39.1	73.5	45.5

CR = Cane rat; SH = spring hare.


2a
2b
3a
3b
4a
4b
5a
5b

Discussion

The body weights of both species were not significantly different but were low (just over 2 kg) compared to the figures of about 4 and 4–7 kg for adult spring hares and cane rats, respectively, quoted by *Walker* [1964]. It is therefore evident that all the animals were juveniles but all at about the same level of maturity, so that comparisons between the species could be made. Furthermore, *Lobley et al.* [1977] found little change in fibre type composition between weaning and early maturity in the rabbit.

The ratio of hind limb muscle weights to body weight is much greater in the spring hare than the cane rat (table I), which is probably to be expected owing to the greater hind limb development in the spring hare (fig. 1). Nearly all the work involved in jumping is done by the extensor muscles of the stifle joint and it is the proportion of these muscles (relative to body weight) which determines, to a great extent, the distance an animal can jump [*Alexander,* 1968]. Using the results of table I (and multiplying by 2 to account for muscles on both sides), the stifle extensors of the cane rat amount to 4.7% of body weight and those of the spring hare to 8.7% of body weight. These figures can be compared with 4.6% for the pig [calculated from the data of *Davies,* 1973], and 9% for the bushbaby [*Alexander,* 1968]. It should be pointed out, however, that other factors, such as skeletal dimensions and tendon lengths, will also affect jumping ability.

Another factor that may affect jumping ability is the proportion of the muscle fibre types in the muscle tissue. The results of this investigation show that, in all four muscles studied, the spring hare had a higher percentage of low SDHase activity muscle fibres (designated here white muscle fibres) than the cane rat. All of these white (anaerobic) muscle fibres would be fast-twitch fibres although the red (aerobic) fibres could be either fast- or slow-twitch [*Davies and Gunn,* 1972]. In any event, the spring hare has relatively more fibres than the cane rat adapted to anaerobic activity which are used for short bursts of activity such as jumping. The cane rat muscles appear to be relatively more adapted to sustained activity such as sustained running. There is therefore a possibility that the proportions of muscle fibre types show some relationship to the locomotory activities, discussed above, of the animals studied.

Another feature of the muscle fibres is that all three fibre types are considerably smaller in diameter in the spring hare than the cane rat for all four muscles studied (table II, fig. 2–5). Several authors, including *Gauthier and Padykula* [1966] on mammals, *George and Naik* [1959] on birds and *Stickland* [1975] on fish, have found a relationship between muscle fibre size and body size in a range of animal species. This relationship appears to hold true in that smaller animals often tend to have a higher proportion of red muscle fibres. In this investigation, however, in which both species have the same body weight, all three muscle fibre types are smaller in the spring hare than the cane rat. In these two rodents, therefore, there appears to be no relationship between muscle fibre size and body size. It is interesting to note, however, that the size of each fibre type in each species is similar in all four muscles. It is possible, therefore, that mean

Fig. 2–5. Photomicrographs of transverse sections of muscle stained for SDHase activity in (a) spring hare and (b) cane rat. × 160. 2 M. biceps femoris. 3 M. gluteus superficialis. 4 M. rectus femoris. 5 M. semimembranosus.

muscle fibre size differences found in different muscles of the same animal [e.g. *Joubert*, 1956; *Staun*, 1963] may be due mainly to the muscles having different proportions of fibre types. As far as the difference in each fibre type size between species is concerned, very little other work has been done on this and so it is difficult to speculate further on this point until more work of this nature has been carried out.

References

Alexander, R. McN.: Animal mechanics (Sidgwick and Jackson, London 1968).

Davies, A.S.: Postnatal development of porcine skeletal muscle; PhD thesis Edinburgh (1973).

Davies, A.S. and Gunn, H.M.: Histochemical fibre types in the mammalian diaphragm. J. Anat. *112:* 41–60 (1972).

Edgerton, V.R. and Simpson, D.R.: The intermediate muscle fiber of rats and guinea pigs. J. Histochem. Cytochem. *17:* 828–858 (1969).

Gauthier, G.F. and Padykula, H.A.: Cytological studies of fiber types in skeletal muscle. A comparative study of the mammalian diaphragm. J. Cell Biol. *28:* 333–354 (1966).

George, J.C. and Naik, R.M.: Studies on the structure and physiology of the flight muscles of birds. 6. Variation in the diameter of the fibres of the pectoralis major and its relation to the muscle size and mode of flight. J. Anim. Morph. Physiol. *6:* 90–94 (1959).

Joubert, D.M.: An analysis of factors influencing postnatal growth and development of the muscle fibre. J. Agric. Sci., Camb. *47:* 59–102 (1956).

Lobley, G.E.; Wilson, A.B., and Bruce, A.S.: An estimation of the fibre type composition of eleven skeletal muscles from New Zealand white rabbits between weaning and early maturity. J. Anat. *123:* 501–513 (1977).

Nachlas, M.M.; Tsou, K.; De Souza, E.; Cheng, C., and Seligman, A.M.: Cytochemical demonstration of succinic dehydrogenase by the use of new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem. *5:* 420–436 (1957).

Padykula, H.A.: The localization of succinic dehydrogenase in tissue sections of the rat. Am. J. Anat. *91:* 107–146 (1952).

Romanul, F.C.A.: Enzymes in muscle. 1. Histochemical studies of enzymes in individual muscle fibers. Archs Neurol., Chicago *11:* 355–368 (1964).

Staun, H.: Various factors affecting number and size of muscle fibres in the pig. Acta Agric. scand. *13:* 293–322 (1963).

Stickland, N.C.: Relationship between size of muscle fibres and body dimensions in a number of teleosts. Experientia *31:* 1279–1281 (1975).

Walker, E.P.: Mammals of the world (Hopkins Press, Baltimore 1964).

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Dr. N.C. Stickland, Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH (UK)

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Comparative aspects of muscle fibre size and succinic dehydrogenase distribution in the longissimus dorsi muscle of several species of East African mammals

N. C. Stickland[1]

Department of Veterinary Anatomy, University of Nairobi, Nairobi, Kenya

Key words. Muscle fibre size · Succinic dehydrogenase · Mammals

Abstract. The distribution of succinic dehydrogenase enzyme activity was investigated in frozen sections of longissimus dorsi muscle taken from several species of East African game animal (giraffe, hartebeest, wildebeest, oryx, gerenuk and dik-dik) as well as local zebu cattle. Muscle fibres were classified as red (high succinic dehydrogenase activity), white (low activity) or intermediate. The mean diameter and percentage distribution of each fibre type were noted as well as the overall mean muscle fibre diameter (MFD) for each species. The diameters of red muscle fibres were found to be between 54 and 62% of the diameters of the white muscle fibres for all species with MFD differences between species being up to over 100%. The variation in MFD was found to be significantly and positively correlated with live weight, when zebu values were omitted. It was found that the variation in MFD was significantly related to the diameter of muscle fibre types and not to the percent distribution of these fibre types.

Introduction

It has been demonstrated by several investigators that the proportions of various muscle fibre types in a given muscle are related to the type of activity which that muscle is required to perform [e.g. *Olson and Swett,* 1966; *Barnard et al.,* 1971; *Burke et al.,* 1971; *Peter et al.,* 1972; *Edstrom and Lindquist,* 1973]. Different muscles having different functions in the same animal therefore have different proportions of muscle fibre types. Studies on the same muscle in different mammalian species are, however, limited to the diaphragm [*Gauthier and Padykula,* 1966; *Davies and Gunn,* 1972] and to some hind limb muscles [*Davies and Gunn,* 1971; *Stickland,* 1978]. In both cases, however, the distribution of the fibre types was probably related to differences in muscle function in the animals studied. It was decided worthwhile, therefore, to investigate the distribution of fibre types in the longissimus dorsi muscle in a range of East African mammals, all of which have only limited vertebral column movement and therefore similar limited activity in this particular muscle. Another reason for using this muscle is that it is, of course, an important meat muscle. The proportion of aerobic fibres in this muscle has a possible relationship with meat quality [*Ashmore,*

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Table I. Proportion and diameter of muscle fibre types as characterised by succinic dehydrogenase distribution, in the longissimus dorsi muscle of several East African mammals

Animal	Approximate live weight kg	Number of animals	Distribution, %			Diameter, μm			
			R	I	W	R	I	W	Mean
Giraffe *(Giraffa camelopardalis)*	500	2	13.7	33.6	52.7	54.7	81.6	100.5	87.9
Hartebeest *(Alcephalus buselaphus)*	135	3	11.1	54.6	34.3	40.6	50.6	65.7	54.7
Wildebeest *(Connochaetes taurinus)*	215	3	18.8	45.3	35.9	41.0	53.3	72.0	57.7
Oryx *(Oryx beisa)*	170	1	20.6	34.6	44.8	56.0	77.3	97.4	81.9
Gerenuk *(Lithocranius walleri)*	30	2	50.5	22.0	27.5	40.6	55.3	70.3	52.0
Dik-dik *(Rhynchotragus kirki)*	10	4	25.8	32.3	41.9	31.9	41.4	51.3	43.1
Zebu *(Bos indicus)*	350	4	28.2	28.0	43.8	40.0	48.1	64.3	52.9

R = Red muscle fibre; I = intermediate muscle fibre; W = white muscle fibre.

1974] und as all the animals used in this study are potential meat producers [*Ledger*, 1967], some useful information on an applied level may be obtained.

Material and methods

Mature adults of several species of East African game animal, listed in table I, were shot (usually a neck shot) in their natural environment in various parts of Kenya. Samples of longissimus dorsi muscle were removed from the region of the thoraco-lumbar junction (dorsolateral border) of each animal immediately after death, and put on ice for the return journey to the laboratory. In the case of the zebu, muscle samples were removed from the carcasses of animals slaughtered at KMC Abattoir, Athi River, Kenya. Small pieces of the muscle samples were placed on a Leitz freezing microtome stage within 2 h of the animal's death in all cases, and rapidly frozen by spraying with dichlorodifluoromethane (Arcton 12, ICI Ltd). 15-μm sections were cut at –20 °C and stained for succinic dehydrogenase activity by the method of *Nachlas et al.* [1957]. Sections were also stained for fat, using Sudan IV.

By means of a Leitz projection microscope, the number of muscle fibres (red, white or intermediate) per square millimetre was counted in the sections stained for succinic dehydrogenase activity. The average size of muscle fibres was estimated by measuring the diameters of 100 fibres of each type, using a calibrated eyepiece graticule. The overall mean fibre diameter (MFD) for each species was calculated from the figures for distribution and size of the three muscle fibre types. It should be noted that the classification of muscle fibres into red, white or intermediate was based on the degree of succinic dehydrogenase activity, red being high and white low.

Although some of the smaller animals could easily be weighed, the live weights of the larger animals noted in table I were based mostly on estimates [from data of *Ledger*, 1967 and others]. The statistical methods were based on *Snedecor and Cochran* [1967].

Table II. Regression lines and correlation coefficients for various parameters detailed in table I

	Regression line	r	p (of r)	r^2, %
MFD (Y) versus live weight (X)[a]	$Y = 32.5 \cdot \sqrt[7.81]{X}$	0.695	< 0.10	48.3
MFD (Y) versus live weight (X)[b]	$Y = 29.2 \cdot \sqrt[6.19]{X}$	0.838	< 0.05	70.3
MFD, at constant percent distribution (Y) versus MFD (X)[b]	$Y = 0.86 \cdot X + 8.43$	0.987	< 0.001	97.4
MFD, at constant fibre type diameters (Y) versus MFD (X)[b]	$Y = 0.11 \cdot X + 55.7$	0.589	> 0.10	34.7

[a] All species.
[b] All species, except the zebu.

Results

All the data obtained for the animals investigated are shown in table I. The fat content was low, especially in the game animal muscles, but the general distribution tended to correspond to the distribution of succinic dehydrogenase activity. Equations of regression lines and correlation coefficients for various parameters are shown in table II. As can be seen from table II, some calculations omitted the data on zebu samples. This was felt to be legitimate and, indeed, necessary as these animals were reared under very different conditions (in a domesticated or semi-domesticated situation) to the wild game animals. With zebu data omitted, there was a significant correlation between MFD and live weight. As red fibres are 54–62% of the diameters of white fibres, the MFD (last column of table I) could be determined by either percentage distribution or size of muscle fibre types or by a combination of both parameters. In order to study this, the significance of correlations was estimated between actual MFD (given in table I) and MFD figures calculated, first, by using average percentage distribution figures for all species with species fibre type diameter figures, and, second, by using average fibre type diameters for all species with species fibre type percentage distribution. The results suggest that MFD for the longissimus dorsi muscle is related far more (up to 97% variation explained) to muscle fibre type sizes than to percentage distribution (only up to 35% variation explained) of muscle fibre types.

Discussion

One of the more obvious features to be noted from the data of table I is that the red fibres are considerably smaller in diameter than the white fibres with the intermediate fibres between the two extremes. This inverse relationship between fibre size and aerobic capacity is well established [e.g. *Goldspink*, 1969]. For all animals studied, the red muscle fibre diameters are within the range of about 54–62% of the white fibre diameters. This is a fairly narrow range when it is considered that the MFD range is from 43.1 μm in the dik-dik to 87.9 μm in the giraffe, i.e. a difference of over 100%.

The more important result in this investigation, however, is the possible relationship

between MFD and body size. It has been reported that there is a relationship between MFD in a given muscle and body size in 13 mammalian species [*Gauthier and Padykula,* 1966], 25 avian species [*George and Naik,* 1959], 13 avian species [*Stickland,* 1977] and 17 fish species [*Stickland,* 1976] as well as several breeds of dogs [*Julian and Cardinet,* 1961]. *Hill* [1956] predicted that MFD should vary as the square root of body size. As linear body size is proportional to $\sqrt[3]{\text{body weight}}$, then this means that MFD α $\sqrt[6]{\text{body weight}}$. In the present investigation there is a very close approximation to this relationship, whence MFD α $\sqrt[6.19]{\text{body weight}}$ (omitting the zebu; table II). The results therefore support *Hill*'s hypothesis.

It is difficult to adequately explain the fact that the same fibre type in different species has different mean diameters (table I) but, nonetheless, it is this fact which results in different MFD, for a given muscle, between species (rather than a difference in fibre type proportions). It is possible, however, that the opposite is true as an explanation for the difference in MFD for different muscles of the same species, i.e. differences in fibre type proportions and not fibre type diameters are responsible [*Stickland,* 1978].

In these discussions, it is important, however, to realise that there are several variables which may affect MFD, including exercise [*Goldspink,* 1964], nutritional level [*Joubert,* 1956; *Staun,* 1963; *Stickland et al.,* 1975] and age [*Joubert,* 1956; *Staun,* 1963; *Rowe and Goldspink,* 1969; *Stickland and Goldspink,* 1973]. Exercise may also affect levels of succinic dehydrogenase enzyme [*Howells and Goldspink,* 1974], thereby influencing muscle fibre type distribution percentages. Owing to the possible effect of these variables, it was felt justified in omitting zebu values in some cases (as in table II) as environmental factors were known to be different for these cattle as compared to the wild environment of the game animals. The sources of variation mentioned may be factors which produced the insignificant relationships between MFD and body size found by some authors [*Joubert,* 1956; *Davies and Gunn,* 1972]. It is also interesting to note that these same authors only investigated 'domesticated' animals in contrast to the present study. It is quite probable that, in selective breeding programmes, different breeds of the same species and similar body weight will have very different MFD, e.g. the Pietrain pig has much larger MFD than Large White pigs [*Dumont and Schmitt,* 1970; *Stickland and Goldspink,* 1977].

On a more applied level, it is known that a lack of aerobic fibres is associated with poorer meat quality [*Ashmore,* 1974]. It would be interesting to investigate the meat quality of the animals used in the present investigation to ascertain whether the proportion of fibre types is indeed related to meat quality in a non-domesticated situation.

References

Ashmore, C.R.: Phenotypic expression of muscle fiber types and some implications to meat quality. J. Anim. Sci. *38:* 1158–1164 (1974).

Barnard, R.J.; Edgerton, V.R.: Furukawa, T., and Peter J.B.: Histochemical, biochemical, and contractile properties of red, white, and intermediate fibres. Am. J. Physiol. *220:* 410–414 (1971).

Burke, R.E.; Levine, D.N.; Zajac, F.E.; Tsairis, P., and Engel, W.K.: Mammalian motor units: physiological-histochemical correlation in three types in cat gastrocnemius. Science *174:* 709–712 (1971).

Davies, A.S. and Gunn, H.M.; A comparative histochemical study of the mammalian diaphragm and m.semitendinosus. J. Anat. *110:* 137–139 (1971).

Davies, A.S. and Gunn, H.M.: Histochemical fibre types in the mammalian diaphragm. J. Anat. *112:* 41–60 (1972).

Dumont, B.-L. and Schmitt, O.: Comparative study of muscular structure in pigs of Large White and Pietrain breed. Annls Génét. Sélect. Anim. *2:* 381–391 (1970).

Edstrom, L. and Lindquist, C.: Histochemical fiber composition of some facial muscles in the cat in relation to their contraction properties. Acta physiol. scand. *89:* 391–503 (1973).

Gauthier, G.F. and Padykula, H.A.: Cytological studies of fiber types in skeletal muscle – A comparative study of the mammalian diaphragm. J. Cell Biol. *28:* 333–354 (1966).

George, J.C. and Naik, R.M.: Studies on the structure and physiology of the flight muscles of birds. 6. Variation in the diameter of the fibre of the pectoralis major and its relation to the muscle size and mode of flight. J. Anim. Morph. Physiol. *6:* 90–94 (1959).

Goldspink, G.: The combined effects of exercise and reduced food intake on skeletal muscle fibres. J. cell. comp. Physiol. *63:* 209–216 (1964).

Goldspink, G.: Succinic dehydrogenase content of individual muscle fibres at different ages and stages of growth. Life Sci. *8:* 781–808 (1969).

Hill, A.V.: The design of muscles. Br. med. Bull. *12:* 165–166 (1956).

Howells, K.F. and Goldspink, G.: The effects of age and exercise on the succinic dehydrogenase content of individual muscle fibres from fast, slow and mixed hamster muscles. Histochemie *38:* 195–201 (1974).

Joubert, D.M.; An analysis of factors influencing post natal growth and development of the muscle fibre. J. agric. Sci., Camb. *47:* 59–102 (1956).

Julian, L.M. and Cardinet, G.H.: Fiber sizes of the biceps brachii muscle of dogs which differ greatly in body size. Anat. Rec. *139:* 243 (1961).

Ledger, H.P.: Wildlife and food production with special reference to the semi-arid areas of tropics and sub-tropics. Wld Rev. Anim. Prod. *3:* 11–37 (1967).

Nachlas, M.M.; Tsou, K.; Souza, E. de; Cheng, C., and Seligman, A.M.: Cytochemical demonstration of succinic dehydrogenase by the use of new p-nitrophenylsubstituted ditetrazole. J. Histochem. Cytochem. *5:* 420–436 (1957).

Olson, C.B. and Swett, C.P., jr.: A functional and histochemical characterisation of motor units in a heterogeneous muscle (flexor digitorum longus) in the cat. J. comp. Neurol. *128:* 475 (1966).

Peter, J.B.; Barnard, R.J.; Edgerton, V.R.; Gillespie, C.A., and Stempel, K.E.: Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. Biochemistry, N.Y. *11:* 2627–2633 (1972).

Rowe, R.W.D. and Goldspink, G.: Muscle fibre growth in five different muscles in both sexes of mice. I. Normal mice. J. Anat. *104:* 519–530 (1969).

Snedecor, G.W. and Cochran, W.G.: Statistical methods; 6th ed. (Iowa State University Press, Ames 1967).

Staun, H.: Various factors affecting number and size of muscle fibres in the pig. Acta Agric. scand. *13:* 293–322 (1963).

Stickland, N.C.: Relationship between size of muscle fibres and body dimensions in a number of teleosts. Experientia *31:* 1279–1281 (1976).

Stickland, N.C.: Succinic dehydrogenase distribution in the pectoralis muscle of several East African birds. Acta zool., Stockh. *58:* 41–44 (1977).

Stickland, N.C.: Muscle weights and succinic dehydrogenase distribution in the limb musculature of two rodents *(Thryonomys gregorianus* and *Pedetes capensis)* with different locomotory habits. Acta anat. *102:* 203–208 (1978).

Stickland, N.C. and Goldspink, G.: A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. Anim. Prod. *16:* 135–146 (1973).

Stickland, N.C.; Widdowson, E.M., and Goldspink, G.: Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscle of pigs. Br. J. Nutr. *34:* 421–428 (1975).

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Dr. N.C. Stickland, Department of Anatomy
Royal (Dick) School of Veterinary Studies
Edinburgh, EH9 1QH (UK)

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The arrangement of muscle fibres and tendons in two muscles used for growth studies

N. C. STICKLAND

Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH

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INTRODUCTION

It has been generally accepted that the total number of muscle fibres in a muscle is fixed by about the time of birth, so that postnatal muscle growth is not associated with any fibre hyperplasia (Rowe & Goldspink, 1969). There may, however, be some fibre hyperplasia in muscles of animals which are relatively immature at birth, such as the rat, in which Rayne & Crawford (1975) found significant fibre hyperplasia in the first few weeks post partum. In relatively long muscles of large animals, postnatal fibre hyperplasia may be attributed to muscle fibres growing into the plane of section (Swatland, 1976) so that, in these cases, the hyperplasia is only apparent. It has been concluded that real fibre hyperplasia ceases prenatally in agricultural animals (Joubert, 1956; Swatland, 1973; Stickland, 1978) and humans (Stickland, 1981). A postnatal increase in muscle fibre number can therefore be explained either by real hyperplasia in immature animals or by growth of intrafascicularly terminating fibres in larger muscles. What is more difficult to explain, however, is the postnatal decrease in muscle fibre number which has been found by some authors. Some of the decreases found have been small and insignificant but recently Layman, Hegarty & Swan (1980) claimed substantial decreases in fibre number of four rat muscles during growth, the largest decrease being 41 % in the soleus muscle. Also, Ihemelandu (1980) claimed a 32 % decrease in the total number of fibres in the pectineus muscle of dogs between 2 and 12 months of age. No definitive explanation has been given for these decreases. Both these studies were based on counts made on sections through the 'belly' of the muscles. It is possible that not all fibres would be included in such a section, or that the fibre architecture of a muscle could change during growth so that an apparent decrease might be observed. Because the arrangement of fibres did not seem to have been studied in any detail in these papers, it was felt worthwhile to investigate the muscle fibre and tendon arrangement in the soleus and pectineus muscles of rats and dogs, respectively, at different ages.

MATERIALS AND METHODS

Soleus muscles were removed post rigor from male rats killed at 6, 21, 70 and 175 days post partum. Pectineus muscles were also removed from newborn male pups and from mature male dogs. All muscle samples then underwent a modification of the technique used by Williams & Goldspink (1971). The muscles were fixed in 10 % formalin for 24 hours, washed in water for 6 hours and left in 30 % nitric acid for two days (pulling apart slightly, after one day, for better penetration) and washed

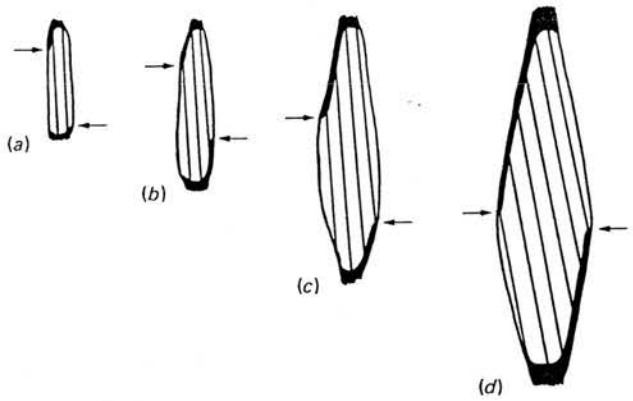

Fig. 1 (*a–d*). The arrangement of representative muscle fibres (thin lines) and tendons (thicker black areas) in the soleus muscles from rats of (*a*) 6 days, (*b*) 21 days, (*c*) 70 days and (*d*) 175 days post partum. The portions between the arrows show where a transverse section would cut all muscle fibres. The tendon of origin is at the top and the tendon of insertion at the bottom for each muscle. All Figures are × 2 actual size.

again in water. The muscles were then transferred to 50% glycerol and, after 24 hours, their muscle fibre architecture was examined by teasing, using fine needles, under a stereo dissecting microscope. Graph paper was placed under the Petri dish in which the muscles were teased so that accurate drawings could be made onto another piece of graph paper. Particular attention was paid to the identification of the extent of tendons and to the distribution of muscle fibre terminations.

RESULTS

Rat soleus muscle

The arrangements of muscle fibres and tendons in the soleus muscles from rats of various ages are shown in Figure 1. At 6 days the muscle exhibited a simple arrangement with muscle fibres running parallel to the long axis of the muscle and all fibres running approximately the full length of the muscle from the tendon of origin to the tendon of insertion. With growth, however, the arrangement was seen to change. The origin and insertion tendons gradually extended along opposite sides of the muscle towards the mid-point so that the number of fibres extending the full length of the muscle diminished. This resulted in the muscle changing to a unipennate structure at 175 days, with muscle fibres running at an angle to the long axis of the muscle. At this stage there was only a short distance (about 7% of the muscle length) in which a section would cut all muscle fibres but, more importantly, this portion was in the distal half of the muscle; a section midway along the muscle length would not cut through all constituent muscle fibres.

Dog pectineus muscle

The arrangements of muscle fibres and tendons in the pectineus muscle from newborn and mature dogs is shown in Figure 2. The pectineus muscle exhibited a bipennate structure which did not appear to change significantly with age. The distal tendon surrounded most of the distal half of the muscle, with muscle fibres inserting into it at an angle along all of its surface. The extent of the tendinous invagination into the belly of the muscle from the proximal tendon was not apparent superficially; it

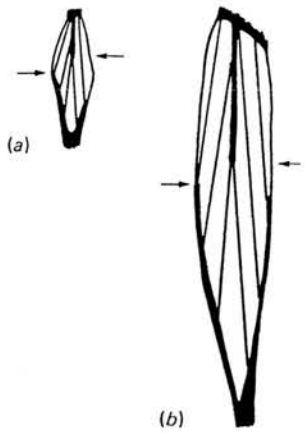

Fig. 2 (*a*, *b*). The arrangement of representative muscle fibres (thin lines) and tendons (thicker black areas) in pectineus muscles from (*a*) newborn and (*b*) mature dogs. The portions between the arrows show where a transverse section would cut all muscle fibres. The tendon of origin is at the top and the tendon of insertion is at the bottom for each muscle. (*a*) is $\times 1\frac{1}{3}$ actual size; (*b*) is $\times \frac{2}{3}$ actual size.

occurred deep in the muscle and was evident only upon teasing. The portion of the muscle through which a transverse section would cut all muscle fibres was only about 13 % of the muscle length in the newborn and only about 5 % in the mature dog. Moreover, although this portion included the mid-length level in the newborn, it did not include the mid-length level in the mature dog.

DISCUSSION

The results have shown that there is a considerable change with age in the muscle fibre and tendon arrangement in the soleus muscle of the rat. Layman *et al.* (1980) claimed a 41 % decrease in the number of fibres in the rat soleus muscle throughout the period of study from 25 to 165 days post partum. The decreases found in the earlier ages are perhaps difficult to explain by the fibre architecture because it would be fairly easy to cut a transverse section through all muscle fibres; the errors are more likely to arise in the later ages. However, the method used by Layman *et al.* (1980) was to cut a transverse section of a thickness which approximates fibre diameter and then to use a Coulter Counter to count the resulting 'fibre cylinders' which are separated by a special technique (Thompson, Levine, Hegarty & Allen, 1979). A comparison of the results obtained by this method with hand counting was carried out by Layman *et al.* (1980) and they stated an error of + 14 % (an over-estimate) at 25 days and − 8 % (an under-estimate) at 165 days. In other words, a fibre decrease of up to 22 % could possibly be explained by an error in the technique. The rest of the decrease (to bring it up to 41 %) could possibly be explained by sections not including all fibres in the muscles from later ages. The arrangement shown in Figure 1 (*d*) for the adult rat soleus muscle was shown by Close (1964) who also examined the arrangement in the rat extensor digitorum longus muscle. Of the four muscles studied by Layman *et al.* (1980) this muscle showed a 25 % decrease in fibre number with age, which was the second largest decrease after that occurring in the soleus muscle. Close showed that this muscle in adult rats had an arrangement similar to that in the soleus muscle, but even more pronounced in that no transverse

section through this muscle could section all its constituent muscle fibres. The results in Figure 1 indicate that it might be possible, with care, to section through all constituent muscle fibres of the soleus muscle at all ages. Kugelberg (1976) sectioned rat soleus muscles at the main entrance of the soleus nerve and obtained sections free from tendon insertions. Total fibre counts were done on magnified photomicrographs of the whole muscle section. He counted the muscle fibres in soleus muscles of 10 rats at each of three ages (2, 5 and 34 weeks) and showed no change in the number of fibres. The mean values were 2840 at 2 weeks, 2857 at 5 weeks and 2913 at 34 weeks. His publication includes photomicrographs of complete sections at 5 weeks and 34 weeks to support his results. The different results of Layman *et al.* (1980) are probably, therefore, due to the method of counting and to the problem of sectioning all fibres at later ages.

Another important aspect which can be appreciated from Figure 1 is the fact that the mean muscle fibre length decreases relative to complete muscle length as growth proceeds; the mean fibre length decreases from about 90 % of muscle length at 6 days to about 60 % at 175 days. Layman *et al.* (1980) assumed that fibre length was equivalent to muscle length so that some of their calculations involving their fibre length estimates (e.g. cytoplasmic volume per nucleus) may be misleading.

The results for the dog pectineus muscle have shown that this muscle is not the simple muscle assumed by Ihemelandu (1980); the fibres do not extend from one end of the muscle to the other. The bipennate structure of this muscle makes it difficult to ensure that a section through it would include all muscle fibres and this appears to be particularly true for the older muscles.

In conclusion, therefore, it would appear that rat soleus muscles and dog pectineus muscles (the two muscles in which the greatest postnatal fibre number decrease has so far been claimed) are not suitable for growth studies of muscle fibre number.

SUMMARY

The arrangement of muscle fibres and tendons was examined in the soleus muscle of rats from 6 to 175 days post partum. The muscle was seen to change from a simple structure, with mean fibre length of approximately 90 % of complete muscle length, to a unipennate structure, with mean fibre length of only about 60 % of muscle length. The dog pectineus muscle was also investigated and found to have a bipennate structure throughout postnatal growth. The arrangement of muscle fibres in both these muscles is such that it might be difficult (particularly in the older animals) to cut a transverse section through all the fibres contained in the muscle; some fibres might not enter the plane of section. Results on muscle fibre number in these muscles at different ages may therefore be misleading.

REFERENCES

CLOSE, R. (1964). Dynamic properties of fast and slow skeletal muscles of the rat during development. *Journal of Physiology* **173**, 74–95.

IHEMELANDU, E. C. (1980). Decrease in fibre numbers of dog pectineus muscle with age. *Journal of Anatomy* **130**, 69–73.

JOUBERT, D. M. (1956). A study of prenatal growth and development in the sheep. *Journal of Agricultural Science* **47**, 382–428.

KUGELBERG, E. (1976). Adaptive transformation of rat soleus motor units during growth. Histochemistry and contraction speed. *Journal of the Neurological Sciences* **27**, 269–289.

LAYMAN, D. K., HEGARTY, P. V. J. & SWAN, P. B. (1980). Comparison of morphological and biochemical parameters of growth in rat skeletal muscles. *Journal of Anatomy* **130**, 159–171.

RAYNE, J. & CRAWFORD, G. N. C. (1975). Increase in fibre numbers of the rat pterygoid muscles during postnatal growth. *Journal of Anatomy* **119**, 347–357.

ROWE, R. W. D. & GOLDSPINK, G. (1969). Muscle fibre growth in five different muscles in both sexes of mice. I. Normal mice. *Journal of Anatomy* **104**, 519–530.

STICKLAND, N. C. (1978). A quantitative study of muscle development in the bovine foetus (*Bos indicus*). *Anatomia Histologia Embryologia* **7**, 193–205.

STICKLAND, N. C. (1981). Muscle development in the human fetus as exemplified by m. sartorius: a quantitative study. *Journal of Anatomy* **132**, 557–579.

SWATLAND, H. J. (1973). Muscle growth in the fetal and neonatal pig. *Journal of Animal Science* **37**, 536–545.

SWATLAND, H. J. (1976). Recent research on postnatal muscle development in swine. *Proceedings of the 29th Annual Reciprocal Meat Conference of the American Meat Science Association*, pp. 86–103.

THOMPSON, E. H., LEVINE, A. S., HEGARTY, P. V. J. & ALLEN, C. E. (1979). An automated technique for simultaneous determinations of muscle cell number and diameter. *Journal of Animal Science* **48**, 328–337.

WILLIAMS, P. E. & GOLDSPINK, G. (1971). Longitudinal growth of striated muscle fibres. *Journal of Cell Science* **9**, 751–767.

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"GIANT" MUSCLE FIBRES IN SKELETAL MUSCLE OF NORMAL PIGS

By

S. E. Handel*

Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh, EH17 7JH, U.K.

and

N. C. Stickland

Department of Anatomy, The Royal Veterinary College, Royal College Street, London, NW1 0TU, U.K.

INTRODUCTION

The occurrence, within porcine skeletal muscle, of "giant" muscle fibres which possess circular and abnormally large transverse sectional areas has been noted by many researchers (Cassens, Cooper and Briskey, 1969; Hendricks, Lafferty, Aberle, Judge and Forrest, 1971; Bader, 1982). Most of the detailed work on giant muscle fibres has been carried out on stress-susceptible pigs which exhibit pale, soft, exudative (PSE) muscle (Cassens *et al.*, 1969; Cooper, Cassens and Briskey, 1969; Dutson, Merkel, Pearson and Gann, 1978). The occurrence of giant muscle fibres has become associated with PSE muscle and consequently little attention has been paid to the giant fibres in normal (stress-resistant) pig muscle, although their occurrence has been mentioned (Cassens *et al.*, 1969; Hendricks *et al.*, 1971).

Dutson *et al.* (1978) pointed out that giant fibres bear a marked resemblance to myofibres within the muscle of dystrophic animals of various species. In particular, muscles from human subjects suffering from Duchenne and Becker-type dystrophy possess fibres with the histological appearance of giant fibres (Schmalbruch, 1982), which makes it feasible to suggest that giant fibres might represent some pathological change within the muscle of the pigs concerned. However, so far, it has not been possible to explain fully the occurrence of these giant muscle fibres and, in fact, information on their structural characteristics is often contradictory or incomplete.

During an investigation of postnatal muscle growth in normal pigs, the occurrence of giant muscle fibres was noted. The opportunity was therefore taken to clarify the histochemical and ultrastructural characteristics of these fibres and to study their postnatal development. The aim of the present investigation was to try to provide some explanation for the existence of giant fibres in apparently normal muscle.

MATERIALS AND METHODS

Muscle tissue samples were prepared for both light and electron microscopy from Large White pigs during the course of an investigation into the postnatal growth and

*Present address: Muscle Biology Laboratory, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

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development of skeletal muscle. All 49 pigs used were from a commercial pedigree herd and ranged in age from birth to 128 days and had liveweights between 0·65 and 49·5 kg.

The animals were slaughtered by exsanguination after electrical stunning. Left-side semitendinosus and trapezius muscles were immediately removed and cleaned. A complete mid-belly section was taken from *M. semitendinosus* and from *M. trapezius* a strip up to 2 cm in length was cut from midway along the caudal edge of the thoracic portion. Fresh frozen, 10 μm thick, serial sections of muscle were cut on a Slee retracting rotary cryostat at −22°C, mounted on coverslips and allowed to thaw and dry out at room temperature before the application of histochemical techniques. Serial muscle sections were stained for the demonstration of alkaline-stable adenosine triphosphatase (Guth and Samaha, 1970), succinate dehydrogenase (Nachlas, Tsou, De Souza, Cheng and Seligman, 1957) and glycogen phosphorylase (Takeuchi, 1956).

Muscle samples for electron microscopy were taken from the deep and superficial portions of *M. semitendinosus* and fixed in 5 per cent glutaraldehyde in 0·1 M sodium cacodylate buffered at pH 7·2 for 2 h. This was followed by 2 washes in buffer, post-fixation in 1 per cent osmium tetroxide in buffer for 1 h, 2 washes in double distilled water and, finally, dehydration through graded acetone. The samples were then embedded in Araldite and sections were cut at a thickness of 60 to 80 nm with a Reichert OMU3 microtome and stained with uranyl acetate (20 min) and lead citrate (4 to 5 min). These ultrathin sections were examined with a Philips 400 electron microscope at an accelerating voltage of 100 KV.

The characteristic size and shape of giant fibres, as described in the literature and illustrated in Fig. 1, enabled their recognition with both the light and electron microscope. Observations were made on the occurrence and histochemical and ultrastructural properties of these giant fibres. Stereological analysis of ultrathin transverse sections of several giant and normal muscle fibres was performed in order to obtain volume fraction estimates for mitochondria, lipid droplets and myofibrils (Eisenberg, Kuda and Peter, 1974; Elias and Hyde, 1980). A few selected blocks from which giant fibres were identified in transverse section were trimmed and reorientated through 90 degrees to enable the preparation of longitudinal sections from the same fibres.

It should be noted that none of the pigs used in this study exhibited signs of stress-susceptibility before slaughter or were the progeny of PSE-muscled pigs.

RESULTS

All recognized giant fibres apparently possessed a strong capacity for oxidative metabolism, as demonstrated by positive staining for succinate dehydrogenase (SDHase), and seemed to exhibit a negligible propensity for glycolytic metabolism, none being found to react positively for glycogen phosphorylase (GPase). Giant fibres could, however, be classified as either alkaline adenosine triphosphatase (ATPase) positive or negative, although the staining of giant fibres was, with either reaction, of greater intensity than in adjacent normal fibres of the same classification (Fig. 1). Of the giant fibres distinguished in *M. semitendinosus*, 64 per cent exhibited "slow" fibre histochemical staining properties while in *M. trapezius* the value was 53 per cent.

In histochemically stained muscle sections, giant fibres could only be distinguished in muscle from pigs of 12 days of age or older because in pigs less than 12 days of age, giant fibres failed to exhibit the large transverse sectional areas and characteristic ATPase staining reactions evident in those of older

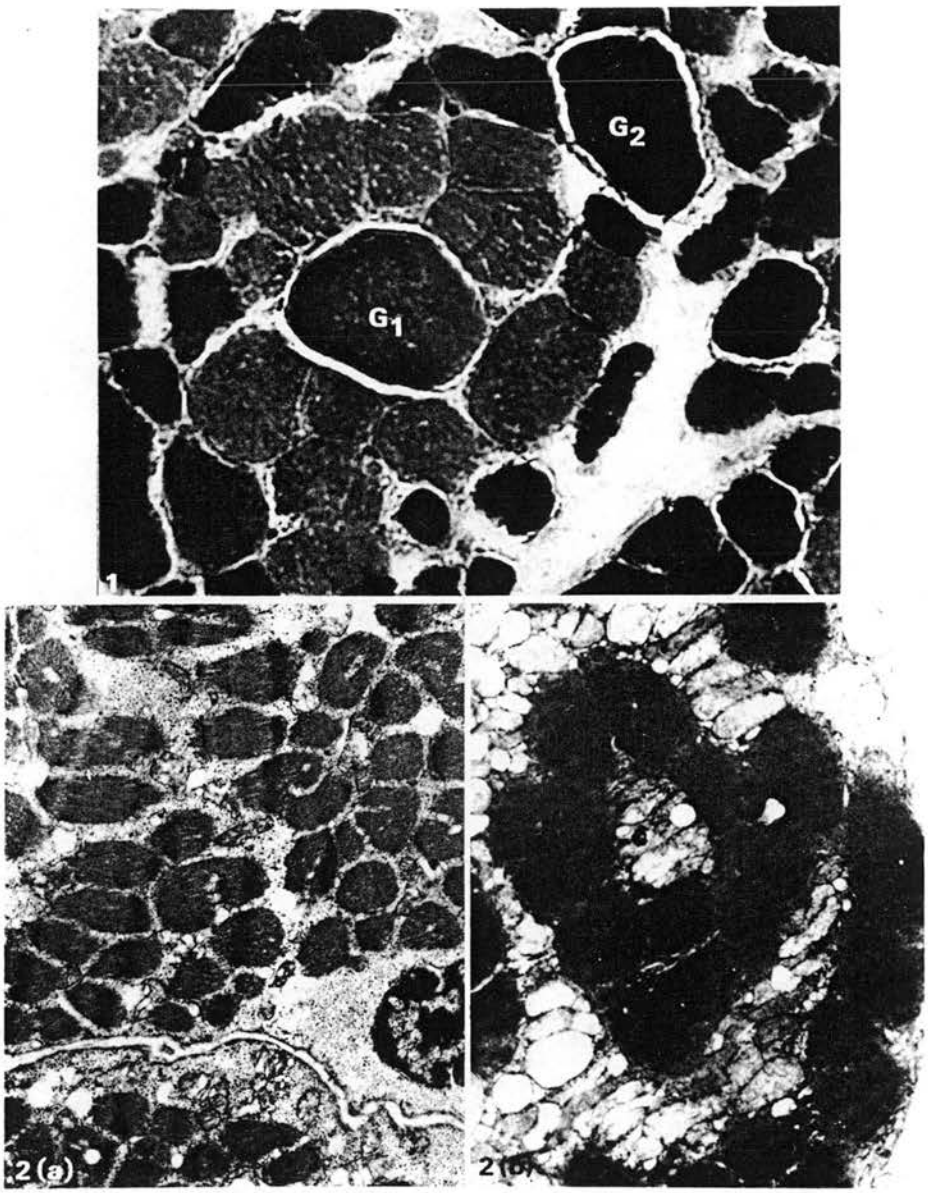

Fig. 1. Alkaline ATPase staining properties of 2 giant fibres (G_1 = alkaline ATPase negative; G_2 = alkaline ATPase positive) from the "deep" portion of *M. semitendinosus* of a 46-day-old pig with a liveweight of 8 kg. × 80.

Fig. 2. Ultrastructural appearance of myofibrils in (a) a normal muscle fibre and (b) a giant muscle fibre from the deep portion of *M. semitendinosus* of a neonatal pig with a liveweight of 0·9 kg. × 6400.

muscle. Giant fibres were seen in the semitendinosus muscle from 17 of the 20 animals (85 per cent) old enough to detect these fibres under the light microscope and 9 of these 17 animals (45 per cent) also possessed giant fibres in their trapezius muscles. In both muscles, under 1 per cent ($0{\cdot}18 \pm 0{\cdot}39$:mean $\pm$ S.D.) of the total myofibre population was classified as "giant" except for 1 pig which contained 2·5 per cent of giant fibres in its *M. semitendinosus* which was the only muscle which bore traits of PSE muscle, i.e. it was abnormally pale and was flabby and watery.

As mentioned above, giant fibres could not be detected by light microscopy in the muscle of pigs less than 12 days of age. However, in transverse sections of neonatal muscle viewed with the electron microscope, giant fibres exhibiting intense myofibrillar staining were discernible [Fig. 2(a) and (b)].

Table 1 displays the percentage volumes occupied by organelles of several giant fibres identified from *M. semitendinosus* and the mean composition of myofibres from the same portion (deep or superficial) of these animals' muscles. Comparison of these results suggested certain trends within the giant fibre ultrastructure. Giant fibres, on average, possessed a greater percentage volume of mitochondria (71 per cent more), lipid droplets (272 per cent more) and myofibrils (18 per cent more) than the normal myofibres, while possessing about 51 per cent less sarcoplasmic reticulum and 65 per cent less remaining sarcoplasm [demonstrated visually in Fig. 3(a) and (b)]. Table 1 also indicates that these ultrastructural features were present at birth, with no substantial changes with subsequent postnatal growth. Higher magnification of transverse sections showed that the regular hexagonal array of myofilaments normally evident in myofibres [Fig. 4(a)] was lacking in the giant fibres [Fig. 4 (b)]. Examination of longitudinal sections of giant fibres revealed that the characteristic myofibrillar banding pattern of skeletal muscle fibres was absent and only one type of broad, kinked band was apparent, the distance between these bands being about $\frac{1}{3}$ the length of the sarcomeres (Z-band to Z-band) in normal fibres [Fig. 3(b)]. Giant fibres were observed in association with evidently normal fibres (i.e. electron density similar to that of other surrounding normal fibres) but the latter appeared to exhibit regions where the

TABLE 1

THE ORGANELLE PERCENTAGE VOLUME COMPOSITION OF GIANT AND NORMAL FIBRES FROM *M. SEMITENDINOSUS* OF A FEW SELECTED ANIMALS

Age of pig in days	*Fibre Type*		*Percentage volume occupied by organelle*				
			Mitochondria	*Lipid droplets*	*Myofibrils*	*Sarcoplasmic reticulum*	*Cytoplasm*
0	Normal	(n = 10)	10·7	3·5	43·2	5·1	38·2
	Giant	(n = 1)	22·0	7·5	56·0	3·0	11·2
4	Normal	(n = 10)	8·1	2·9	39·6	7·1	42·3
	Giant	(n = 1)	11·2	7·3	68·1	4·0	9·4
33	Normal	(n = 10)	4·1	1·9	72·5	10·3	11·2
	Giant	(n = 4)	11·6	9·6	62·5	5·2	8·5
84	Normal	(n = 10)	11·3	1·8	53·7	7·9	25·3
	Giant	(n = 2)	13·6	13·3	58·1	2·8	12·2

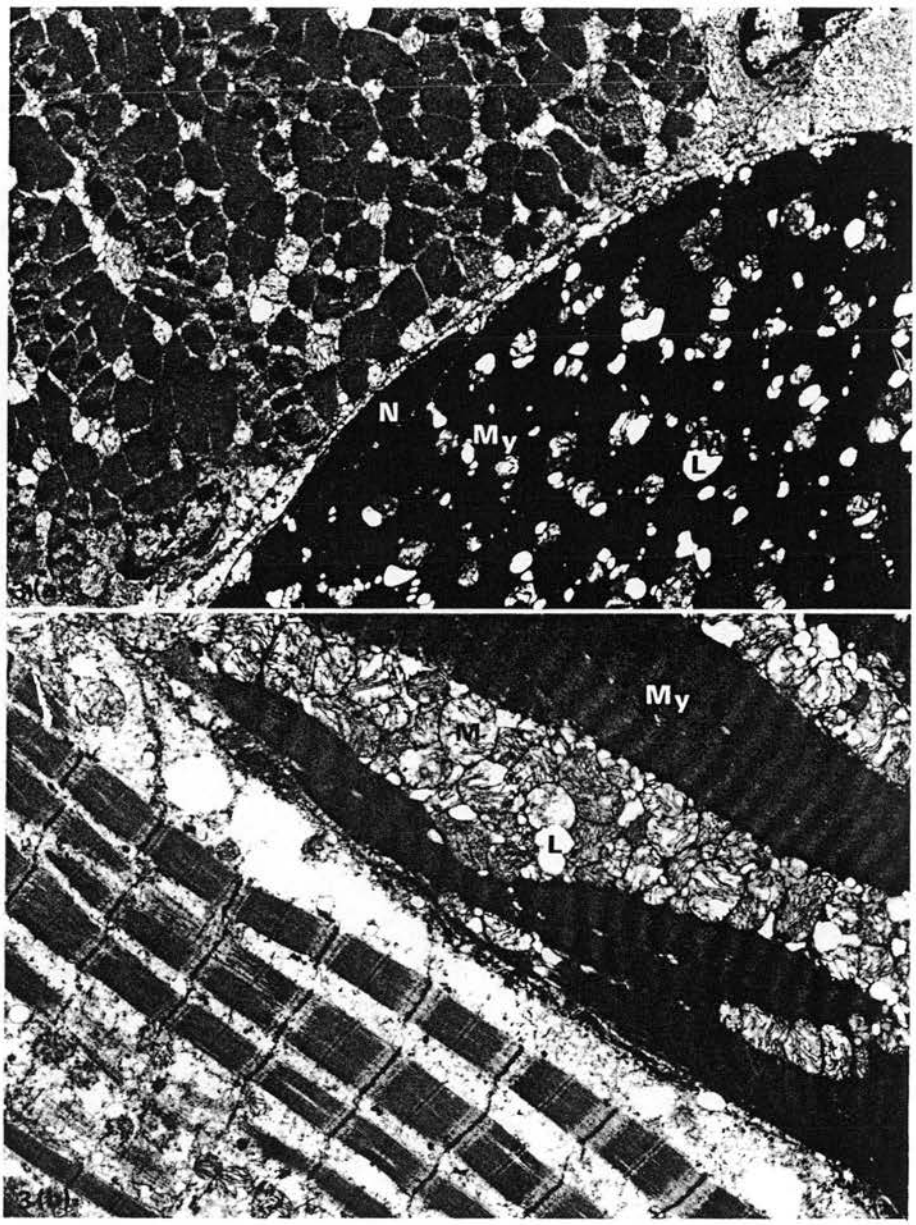

Fig. 3. Ultrastructural appearance of a giant fibre with an adjacent normal muscle fibre in (a) transverse section (×5300) and (b) longitudinal section (×6200). These muscle samples were taken from the deep portion of *M. semitendinosus* of an 84-day-old pig with a liveweight of 27 kg. Note the abundant mitochondria (M) and lipid droplets (L), and the greater electron density of the flattened nucleus (N) and myofibrils (My) of the giant fibre.

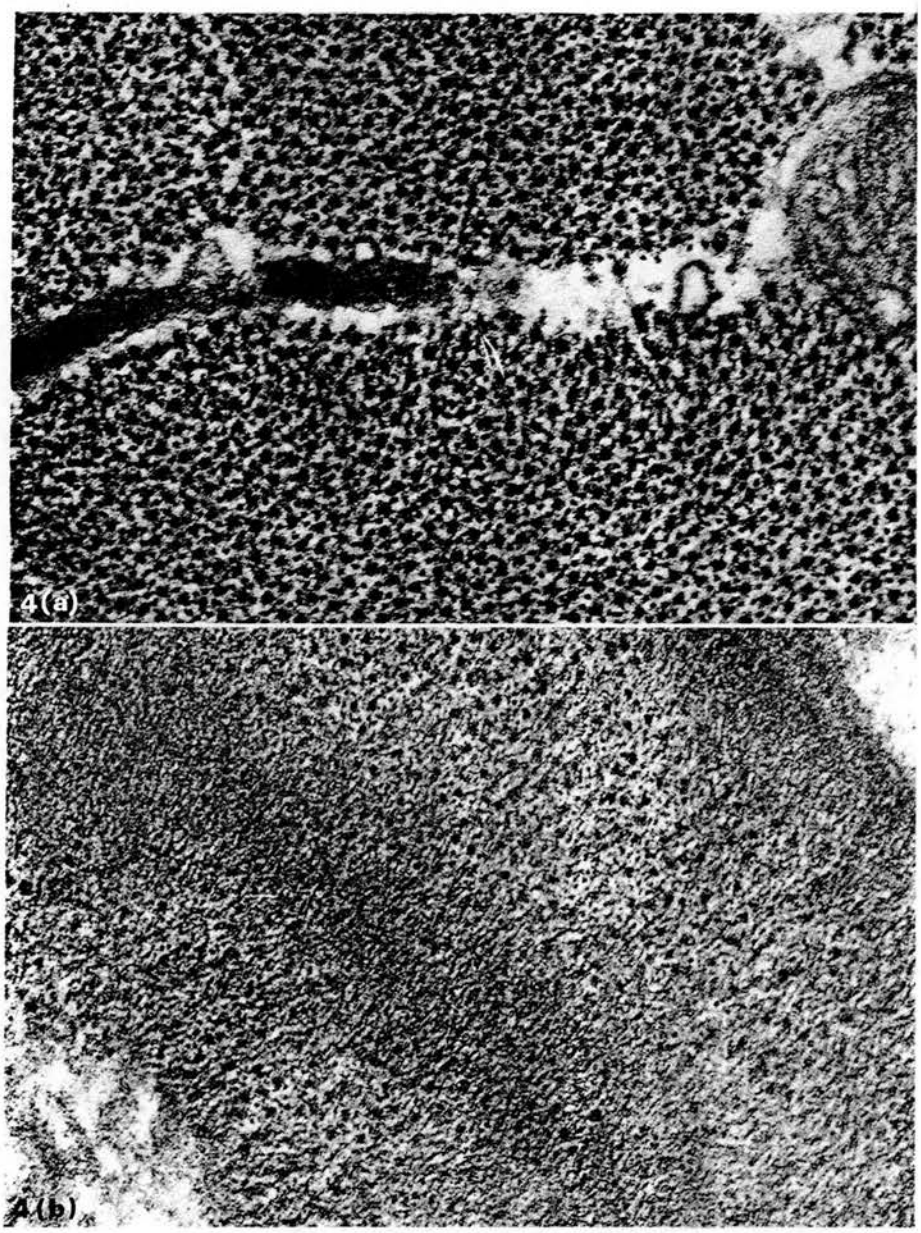

Fig. 4. Transverse section to illustrate myofilament arrangement of (a) normal and (b) giant fibres. The myofilaments of giant fibres failed to exhibit the characteristic hexagonal array evident in normal fibres. × 99 500.

sarcomere length was reduced and the normal banding pattern had been obliterated (Fig. 5).

DISCUSSION

Although giant fibres have been exclusively associated with muscle from low quality, stress-susceptible pigs by some workers (Cooper *et al.*, 1969; Dutson *et al.*, 1978), they have also been observed in the muscle of pigs not exhibiting such conditions (Cassens *et al.*, 1969; Hendricks *et al.*, 1971). Nevertheless, no workers have reported such a high incidence of normal pigs possessing these fibres as was determined in the present study. In the results presented here, 85 per cent of animals of 12 or more days of age were shown to possess giant fibres in their muscles, compared with previous reports of 8 per cent (Cassens *et al.*, 1969) and 30 per cent (Dutson *et al.*, 1978).

Both Dutson *et al.* (1978) and Cassens *et al.* (1969) found that when giant fibres occurred they represented, as determined in the present study, less than 1 per cent of the total myofibre population. However, the former authors obtained this value from low quality PSE Yorkshire pig muscle, whereas the muscle in the present study and that of Cassens *et al.* (1969) was not known to be of poor quality. It is perhaps feasible to suggest that the apparent relationship between giant fibres and PSE muscle is merely a consequence of both of them being enhanced through inbreeding and being more prevalent in certain breeds of pig. Further evidence was obtained from the histochemical

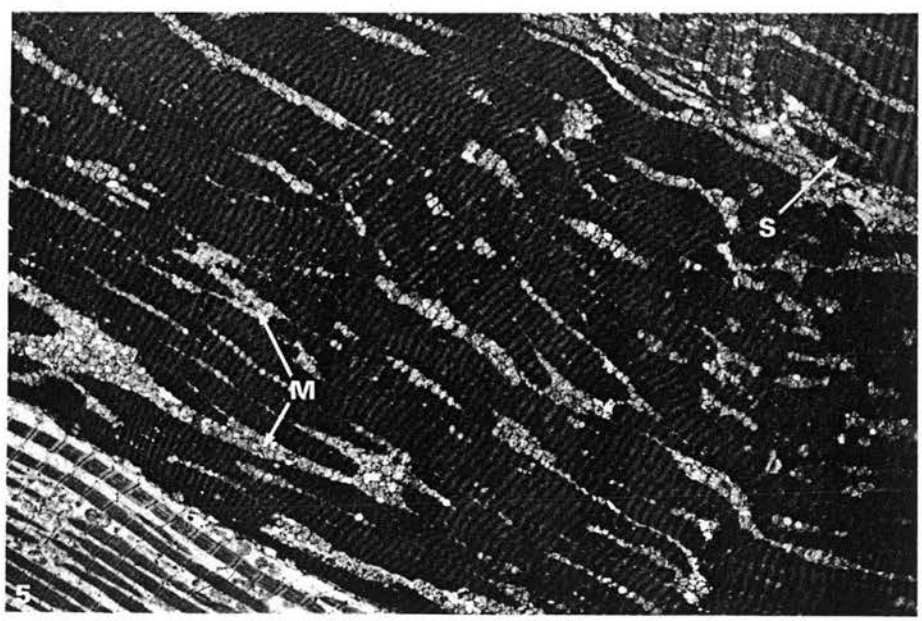

Fig. 5. Longitudinal section of a giant fibre (centre) and adjcent normal fibres. Portions of the normal fibres often exhibited areas of supercontraction (S) but failed to show the intense electron density of myofibrils of giant fibres. Note high concentration of mitochondria (M) throughout the giant fibre. × 1900.

observations on giant fibres for the idea that the association between giant fibres and PSE muscle is not "real".

Giant fibres have been found by some workers to be exclusively alkaline ATPase positive (Cassen *et al.*, 1969) or, when histochemical techniques were not performed, the giant fibres are peripherally located within fascicles (Hendricks *et al.*, 1971; Van Den Hende, Muylle, Oyaert and De Roose, 1972), the corresponding location of histochemically defined fast fibres. Although, more recently, Bader (1982) found giant fibres that exhibit negative alkaline ATPase staining and Dutson *et al.* (1978) found that giant fibres are not confined to the outer positions within fascicles, it appears a somewhat novel discovery that as high a proportion as over half of the giant fibre population classified showed slow staining properties.

Although giant fibres of the present study were found to exhibit high oxidative staining, the majority have previously been shown to possess a range in activity from positive to near negative (Cassens *et al.*, 1969; Dutson *et al.*, 1978). The glycolytic capacity of giant fibres has only been defined in the literature by Cassens *et al.* (1969) whose work supports the view of the present study that these fibres exhibit a neglible propensity for glycolytic metabolism, as would be expected to coincide with the apparently high capacity for oxidative metabolism.

These histochemical properties of giant fibres do not tally with the concept of an association between giant fibres and PSE muscle which is characterized by the presence of a high proportion of "intermediate" (Cooper *et al.*, 1969) or "white" (Swatland and Cassens, 1973) fibres that give a positive reaction for GPase. If the phenomenon of PSE muscle is elicited after death as a consequence of its possessing a high percentage of glycolytic fibres, giant fibres cannot be a contributor to this problem since their metabolism favours oxidation.

The disarray of myofilaments seen from both transverse [Fig. 4(b)] and longitudinal (Fig. 5) sections of giant fibres under the electron microscope was suggestive of super-contracted myofibres. Super-contraction would, as Dutson *et al.* (1978) proposed, explain the lack of characteristic myofibrillar banding, the thickness and kinking of the bands evident in longitudinal section and the absence of the hexagonal orientation of myofilaments in transverse section. However, despite the similarities between the giant fibres of Dutson *et al.* (1978) and those of the present study in this respect, the giant fibres observed by Dutson *et al.* (1978) apparently did not contain any recognizable mitochondria or sarcoplasmic reticulum, while those of the present study clearly exhibited an excessive number of mitochondria in a well-preserved state [Fig. 3(a) and (b)]. It is probable that giant fibres are more susceptible to post-mortem lysis and that the exceptionally long, (24 h) lapse between death and muscle fixation in the study of Dutson *et al.* (1978) was more than adequate to allow this breakdown and cause the numerous disrupted mitochondria and sarcoplasmic reticulum to appear as the vacuolar structures which these workers could not explain. Evidence for the concept that the subcellular components of abnormal muscle fibres are more susceptible to post-mortem disruption is also provided by the observations made by Cloke,

Davis, Gordon, Hsieh, Grider, Addis and McGrath (1981) on the effects of severe freeze-thaw contraction on muscle samples from normal and PSE porcine muscle; the membrane-bound organelles of PSE muscle were markedly disrupted after this treatment, indicating their relatively enhanced fragility.

Functionally-stressed myofibres are also more prone to post-mortem lysis. Schmerling, Filyushina and Buzueva (1981) found that the anterior tibialis muscle of rats which had been subjected to intense physical work to the point of fatigue showed a disturbed myofibrillar organization and disrupted mitochondria. This finding might also relate to the apparent absence of mitochondria in the giant fibres observed by Dutson *et al.* (1978) as it is obvious from the ultrastructural investigations performed in the present study that the giant fibres are in fact functionally overloaded. The greater oxidative capacity of these fibres, as recognized by the greater percentage volume of mitochondria and lipid droplets (as well as high SDHase activity) than in normal fast or slow myofibres, is consistent with the changes seen within muscle fibres subjected to prolonged contraction. The fast myofibres of genetically spastic mice, a condition that subjects the muscles to increased nervous activity, are seen to adapt to the almost continual stimulation by an increased capacity for oxidative metabolism (Levin, Degennaro, Ross, Serafin and Stewart, 1981). The increased activity of muscle induced by exercise (as reviewed by Salmons and Henriksson, 1981) is associated with an increased mitochondrial volume, significantly above that of control fast and slow muscles and also dramatically reduced sarcoplasmic reticulum, which considerably affects calcium ion uptake from the myofibres and consequently muscle relaxation.

Although giant fibres have been compared with myofibres of certain muscular dystrophies (Dutson *et al.*, 1978), such myopathies are invariably associated with degenerative changes (Shafiq, Gorycki and Milhorat, 1969; Schmalbruch, 1982), no signs of which were evident in the giant fibres of the present investigation.

The observations of metabolic changes associated with combating the effects of fatigue support the idea that the structural abnormalities of giant fibres are induced by functional overload associated with sustained, high intensity contraction. The super-contraction of giant fibres was occasionally observed to elicit a similar hyper-contractile effect in adjacent fibres (Fig. 5) which, due to its inconsistency along the length of the myofibre, suggests that it was possibly a passive effect generated by physical contact with the giant fibres. Super-contracted portions of normal fibres did not possess myofibrillar electron density as great as that seen in giant fibres (Fig. 5). This phenomenon, together with the abnormally high ATPase activity (whether alkaline stable or labile) of the giant fibres, suggests that the extent of super-contraction within giant fibres was sufficiently chronic to induce compensatory myofibrillar proliferation and enhanced ATPase activity.

It is therefore suggested that giant fibres, since they were present in the muscle at birth [Fig. 2(b)] and failed to exhibit any marked ultrastructural changes after birth (Table 1), perhaps resulted from some defect in developing muscle fibres such as an inadequate amount of sarcoplasmic reticulum, eliciting hypercontractile activity and consequential structural and metabolic

anomalies within the fibres. Giant fibres do not appear to be a result of degenerative changes within the muscle.

SUMMARY

Observations made during a growth and development study of the semitendinosus and trapezius muscles of 49 purebred Large White pigs between birth and 128 days of age revealed the presence of giant fibres. The occurrence, histochemical and ultrastructural properties of these giant fibres were investigated. A high proportion of the pigs (85 per cent) contained giant fibres in their muscles but these giant fibres usually represented less than 1 per cent of the total myofibre population. Giant fibres possessed enhanced adenosine triphosphatase activity and a high capacity for oxidative metabolism (indicated by succinate dehydrogenase activity) which was reflected ultrastructurally by the greatly heightened electron density of myofibrils and by an abnormally high percentage of mitochondria and lipid droplets. These deviations from normal muscle fibre composition, together with the reduced percentage volume of sarcoplasmic reticulum, were consistent with changes seen in functionally over-loaded muscle. It appears that giant fibre anomalies occur through increased activity stimulated in occasional muscle fibres, perhaps by a structural defect, such as an inadequate amount of sarcoplasmic reticulum, which causes hyper-contractile activity within the fibres and associated compensatory adaptations. Giant fibres did not appear to represent fibres undergoing degenerative changes.

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REFERENCES

Bader, R. (1982). Histologische Befunde aus licht- und elektronenmikroskopischen Untersuchungen an der Skelettmuskulatur von gesunden, ausgemasten Schweinen der Deutschen Landrasse. *Zentralblatt für Veterinärmedizin, A*, **29,** 458–476.

Cassens, R. G., Cooper, C. C. and Briskey, E. J. (1969). The occurrence and histochemical characterisation of giant fibres in the muscle of growing and adult animals. *Acta Neuropathologica*, **12,** 300–304.

Cloke, J. D., Davis, E. A., Gordon, J., Hsieh, S-I., Grider, J., Addis, P. B. and McGrath, C. J. (1981). Scanning and transmission electron microscopy of normal and PSE porcine muscle. *Scanning Electron Microscopy*, **3,** 435–446.

Cooper, C. C., Cassens, R. G. and Briskey, E. J. (1969). Capillary distribution and fibre characteristics in skeletal muscle of stress-susceptible animals. *Journal of Food Science*, **34,** 299–302.

Dutson, T. R., Merkel, R. A., Pearson, A. M. and Gann, G. L. (1978). Structural characteristics of porcine skeletal muscle giant myofibres as observed by light and electron microscopy. *Journal of Animal Science*, **46,** 1212–1220.

Eisenberg, B. R., Kuda, A. M. and Peter, J. B. (1974). Stereological analysis of mammalian skeletal muscle. *Journal of Cell Biology*, **60,** 732–754.

Elias, H. and Hyde, D. M. (1980). An elementary introduction to stereology (quantitative microscopy). *American Journal of Anatomy*, **159,** 412–446.

Guth, L. and Samaha, F. J. (1970). Research note: procedure for the demonstration of actomyosin ATPase. *Experimental Neurology*, **28,** 365–367.

Hendricks, H. B., Lafferty, D. T., Aberle, E. D., Judge, M. D. and Forrest, J. C. (1971). Relation of porcine muscle fibre type and size to postmortem shortening. *Journal of Animal Science*, **32,** 57–61.

Levin, M. A., Degennero, P., Ross, A., Serafin, N. and Stewart, J. A. (1981). A histochemical and electron microscopic study of the fast- and slow-twitch muscle in genetically spastic mice. *Tissue and Cell*, **13,** 61–69.

Nachlas, M. M., Tsou, K., De Souza, E., Cheng, C. and Seligman, A. M. (1957). Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *Journal of Histochemistry and Cytochemistry*, **5,** 420–436.

Salmons, S. and Henriksson, J. (1981). The adaptive response of skeletal muscle to increased use. *Muscle and Nerve*, **4,** 94–105.

Schmalbruch, H. (1982). The muscular dystrophies. In *Skeletal muscle pathology*, F. C. Mastaglia and J. Walton, Eds, Edinburgh, London, Melbourne and New York: Churchill Livingstone.

Shafiq, S. A., Gorycki, M. A. and Milhorat, A. T. (1969). An electron microscope study of fibre types in normal and dystrophic muscles of the mouse. *Journal of Anatomy*, **104,** 281–293.

Schmerling, M. D., Filyushina, E. E. and Buzueva, I. I. (1981). Ultrastructural changes in the skeletal muscle fibres under the effects of acute physical stress. *Arkhiv Anatomii Gistologii i Embryologii*, **80,** 43–49.

Swatland, H. J. and Cassens, R. G. (1973). Observations on the postmortem histochemistry of myofibres from stress-susceptible pigs. *Journal of Animal Science*, **37,** 885–891.

Takeuchi, T. (1956). Histochemical demonstration of phosphorylase. *Journal of Histochemistry and Cytochemistry*, **4,** 84.

Van Den Hende, C., Muylle, E., Oyaert, W. and De Roose, P. (1972). Changes in muscle characteristics in growing pigs. *Zentralblatt für Veterinärmedizin, A*, **19,** 102–110.

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The numbers and types of muscle fibres in large and small breeds of pigs

N. C. STICKLAND AND S. E. HANDEL

Department of Anatomy, The Royal Veterinary College, Royal College Street, London NW1 0TU

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INTRODUCTION

The biphasic theory of muscle development is well established for the pig (Ashmore, Addis & Doerr, 1973; Swatland & Cassens, 1973; Beermann, Cassens & Hausman, 1978). Initially, a population of large primary myofibres forms within the presumptive muscle by fusion of myoblasts. The next phase of development is the formation of smaller, secondary, myofibres; these myofibres form by fusion of myoblasts which line up on the surface of the primary myofibres. In mixed fibre type muscles of pigs, it has been shown that primary myofibres take on slow-contracting characteristics whereas secondary myofibres acquire fast-contracting characteristics (Ashmore *et al.* 1973), although some secondaries become slow during late prenatal (Beermann *et al.* 1978) and postnatal growth (Davies, 1972). In postnatal porcine muscle these 'metabolic bundles', derived from one primary myofibre and its surrounding secondaries, can be readily identified (Fig. 1) using methods for the detection of myosin adenosine triphosphatase (myosin ATPase).

Restricted nutritional levels fed to pregnant animals of various species have been found to cause a significant reduction in myofibre number within muscles of the offspring (Everitt, 1968: sheep; Robinson, 1969: pigs; Aziz-Ullah, 1974: mice; Bedi *et al.* 1982: rats). It has been shown by Wigmore & Stickland (1983) that pigs which develop at disadvantaged sites in the uterus often develop fewer myofibres in their muscles and do so because fewer secondary myofibres form around each primary; the number of primary myofibres is not affected. The pig developing at a disadvantaged uterine site is analogous to the undernourished animals in the situations mentioned above.

The present study was carried out in order to determine whether genetically small animals develop fewer muscle fibres in their muscles by the same mechanism as in nutritionally small animals. It is known that selection for small body size in mice results in a decrease in the total number of muscle fibres in a given muscle (Luff & Goldspink, 1967; Hanrahan, Hooper & McCarthy, 1973). Smaller breeds of the same species also have fewer myofibres in their muscles than the larger breeds (Smith, 1963: chickens; Stickland & Goldspink, 1973: pigs). However, the developmental mechanisms responsible for these differences in myofibre number and the consequences upon the proportions of myofibre types in the adult are unknown. The pig provides an ideal model for investigating the proportions of fibre types in postnatal muscle and, for the reasons already discussed, the distribution pattern of myofibre types reflects the developmental stages of myogenesis in terms of primary and secondary myofibres. The numbers, ratios and distribution of muscle fibre

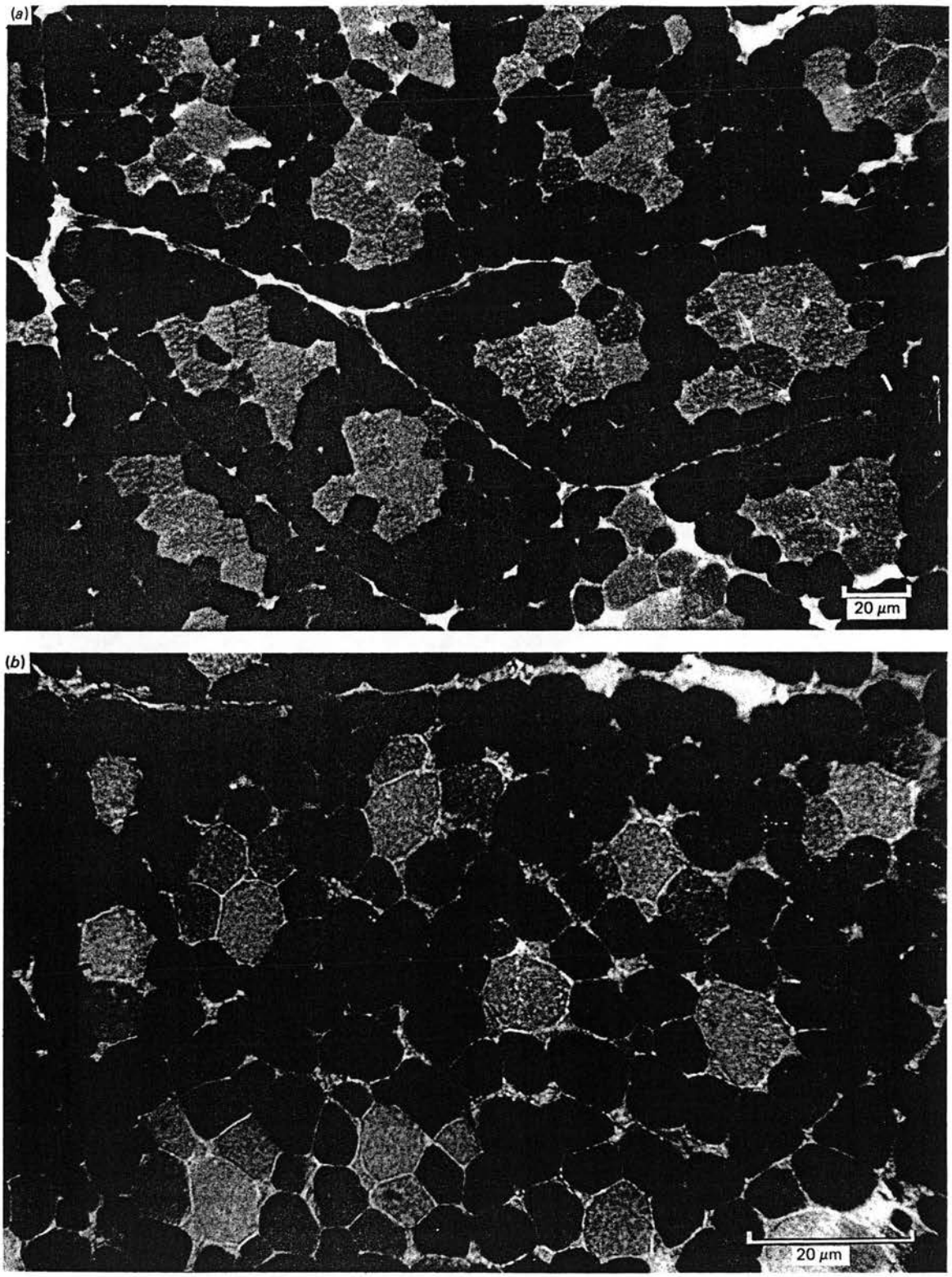

Fig. 1 (*a–b*). Transverse sections of m. semitendinosus reacted for alkaline stable myosin ATPase activity to show the total number of myofibres and the number of slow myofibres in each 'metabolic bundle' in (*a*) Large White pig, 64 days; (*b*) Miniature pig, 61 days.

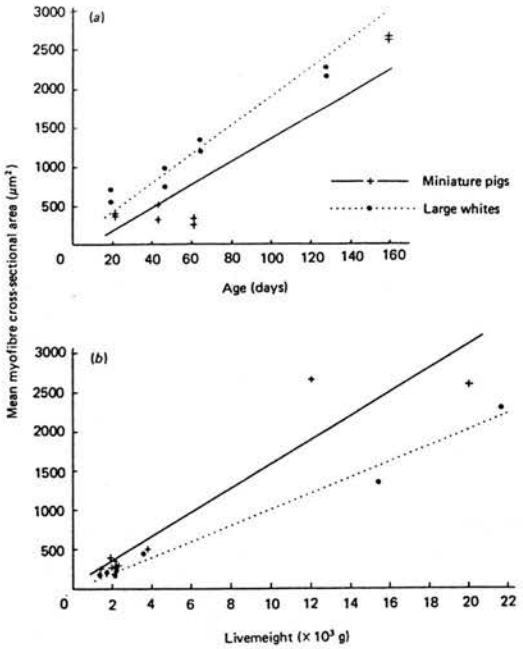

Fig. 2(*a–b*). Mean myofibre cross sectional area plotted against (*a*) age and (*b*) live weight for both miniature pigs and Large White pigs.

types (based mainly on myosin ATPase activity) were therefore investigated in Large White and in miniature pigs; at six months of age the latter are about one third of the body weight of the former, commercial, pigs.

MATERIALS AND METHODS

The miniature pigs were obtained from those reared at the Royal Veterinary College farm (Hawkshead, Herts) which are Göttingen miniature pigs (developed by Haring, Gruhn, Smidt & Scheven, 1966). Eight miniature pigs were used which ranged in age from 21 to 160 days; this corresponded to a weight range from about 2 to 20 kg. Sixteen Large White pigs (from a commercial, pedigree herd) were used, such that eight of them matched the live weights of the miniature pigs and eight matched their ages as closely as possible. The actual ages and live weights may be ascertained from Figure 2.

The pigs were killed by an intraperitoneal injection of pentobarbitone (Euthesate) followed by exsanguination. M. semitendinosus was dissected out from each animal and a complete transverse slice 3–4 mm thick was taken from the muscle, frozen in dichlorodifluoromethane (Arcton 12, ICI Ltd) cooled to its melting point of −158 °C with liquid nitrogen, and frozen sections 10 μm thick taken so that the constituent muscle fibres were cut transversely in a cryostat. A sample of m. trapezius was also taken from midway along the caudal border of its thoracic portion and transverse sections of its muscle fibres were made. Some sections for each muscle sample were treated by the method outlined by Guth & Samaha (1970) for the detection of both alkali-stable and acid-stable myosin ATPase, although the former was used for most of the analysis as it appeared to produce the best differential staining. Sections were,

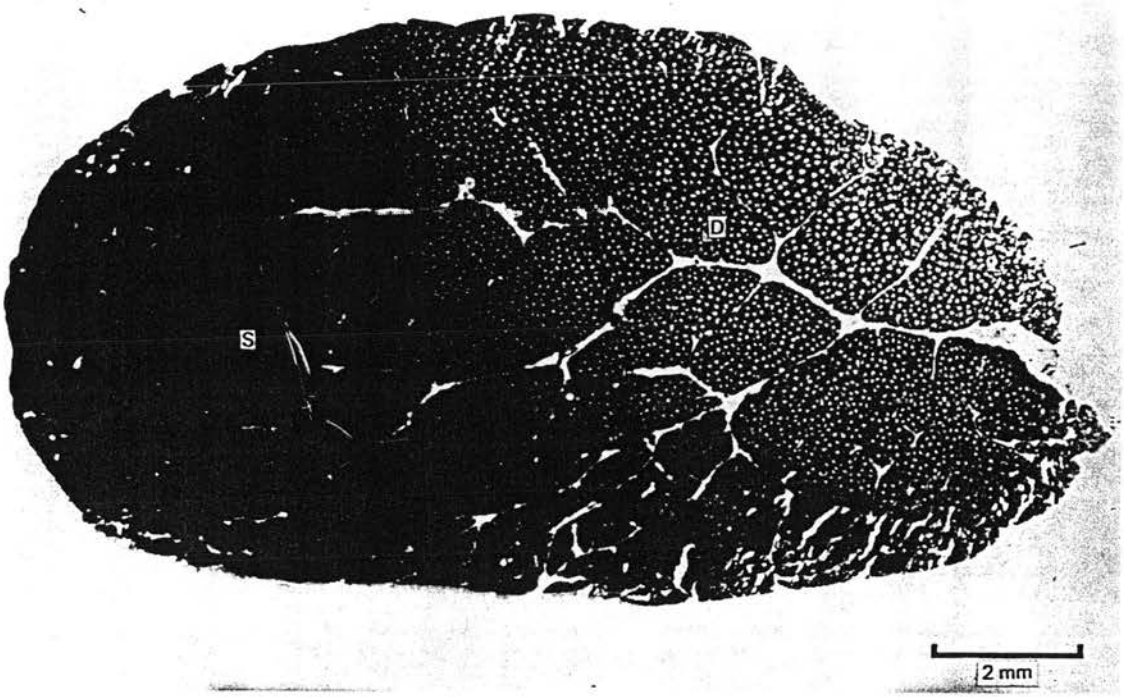

Fig. 3. Complete transverse section of m. semitendinosus reacted for alkaline stable myosin ATPase activity to show the superficial (S) and deep (D) regions of the muscle. Miniature pig, 61 days.

however, also tested for succinate dehydrogenase activity and glycogen phosphorylase activity by the methods of Nachlas *et al.* (1957) and Takeuchi (1956) respectively.

The sections were viewed and analysed using a microscope with camera linked to an image analysis system based on an Apple IIe computer with TV monitor and graphics tablet (VIDS II from Analytical Measuring Systems Ltd, Saffron Walden, Essex). Most of the analysis outlined below was carried out on the alkali-stable myosin ATPase reacted sections for the detection of slow and fast contracting myofibres, with other sections being used for confirmation. Measurements were made on five randomly selected areas of known size (each containing about 500 myofibres) from each muscle. For each area the total number of myofibres was noted as was the number of 'metabolic bundles' (indicated by the number of clusters of slow myofibres). These data were used to estimate the secondary: primary myofibre ratio (equivalent to the number of myofibres per 'metabolic bundle' minus one) for both m. semitendinosus and m. trapezius. In addition, the total cross sectional area of the whole m. semitendinosus was measured so that, for this muscle, the total number of primary myofibres (equal to the number of 'metabolic bundles') and of all myofibres per complete cross section could be estimated. The mean cross sectional area for all myofibres in each muscle was also estimated by measuring the area of fibres from complete 'metabolic bundles' taken from each of the five muscle areas selected such that 100 myofibres were measured for each muscle.

Under low magnification it was seen (Fig. 3) that m. semitendinosus could be divided into a distinguishable superficial region and a deep region; the former region

Table 1. *Numerical data on muscle fibres in the muscles of Large White (age controls) and miniature pigs (means* ± S.E.)

Number of animals	Large White 8	Miniature 8	Significance of difference
M. semitendinosus			
Total myofibre number	392159 (±13956)	143825 (±15286)	$P < 0.001$
Total 'primary' myofibre number	15454 (±791)	7184 (±632)	$P < 0.001$
Secondary:primary myofibre ratio	24·6 (±0·8)	18·9 (±1·0)	$P < 0.001$
M. trapezius			
Secondary:primary myofibre ratio	23·2 (±1·2)	18·3 (±1·1)	$P < 0.02$

contained larger clusters of slow myofibres. The relative proportions of these two areas were estimated for each of the m. semitendinosus sections. Within the deep area of the muscle the number of slow myofibres per 'metabolic bundle' was noted (mean for 100 bundles) as was their mean cross sectional area (based on measurements of 100 slow myofibres). The percentage of the muscle cross sectional area occupied by slow myofibres could therefore be calculated. The number of slow myofibres per 'metabolic bundle' was also noted for m. trapezius.

For most comparisons between the two breeds of pigs the age-control Large Whites were used. The weight-control Large Whites were only used for the analysis of myofibre size.

RESULTS

As there was no evidence of any change in myofibre numbers with growth, the data on cell numbers could be grouped as shown in Table 1. It can be seen from this Table that both total myofibre number and the total primary myofibre number in m. semitendinosus were greater (173 and 115 % respectively) in Large White than in miniature pigs. The secondary:primary myofibre ratio was also greater in both muscles studied in the Large White pigs. However, the results indicate that the primary myofibre number difference was about four times more important than the secondary:primary myofibre ratio difference in bringing about the total myofibre number difference between the two breeds in m. semitendinosus. Although total myofibre number could not be estimated for m. trapezius, the secondary:primary myofibre ratio difference between Large White and miniature pigs was of the same order for m. trapezius (27 %) as that for m. semitendinosus (30 %). It therefore follows that, if the myofibre number difference was the same in m. trapezius, then there must have been a similar difference in primary myofibre number.

The relationship between the mean size of all myofibres and live weight and age are shown in Figure 2 and Table 2. No significant difference between Large White and miniature pigs could be demonstrated in the myofibre cross sectional area changes with age; at any given age mean myofibre areas were not significantly different between the two strains of pigs. However, when mean myofibre area was plotted against live weight the slope or regression coefficient was significantly larger ($P < 0.05$) for the miniature pigs; at any given live weight, the mean myofibre area in the miniature pigs was approximately 63 % greater than in Large White pigs throughout the weight range studied.

For the age range under study there was no evidence of any change with increasing age in the number of slow myofibres per 'metabolic bundle'. The percentage of

Table 2. *Equations for the regression lines shown in Figure 2 of mean myofibre cross sectional area (μm^2) against (a) age and (b) live weight*

(*a*) Against age (in days)		
		SE_b
Miniature pigs	$Y = 14{\cdot}79X - 120$	1·66
Large White pigs	$Y = 18{\cdot}26X + 75$	0·88
(Neither slopes nor intercepts are significantly different.)		
(*b*) Against live weight (in g)		
		SE_b
Miniature pigs	$Y = 0{\cdot}155X + 52{\cdot}57$	0·016
Large White pigs	$Y = 0{\cdot}102X + 17{\cdot}62$	0·004
(Slopes significantly different, $P < 0{\cdot}05$.)		

Table 3. '*Slowness*' *of muscles of Large White (age controls) and miniature pigs (means* ± S.E.)

Number of animals	Large White 8	Miniature 8	Significance of difference
M. semitendinosus			
Deep area as % of whole	39·9 (±1·9)	48·4 (±3·3)	$P < 0{\cdot}05$
Number of slow myofibres per bundle in deep area	8·68 (±0·75)	1·67 (±0·28)	$P < 0{\cdot}001$
Total area of slow myofibres as % of whole muscle area	18·05 (±3·62)	6·18 (±0·77)	$P < 0{\cdot}01$
M. trapezius			
Number of slow myofibres per bundle	9·76 (±0·75)	4·73 (±0·51)	$P < 0{\cdot}001$

muscle cross sectional area occupied by slow myofibres was found to be about three times greater in the Large Whites than the miniature pigs (Table 3). This considerable difference was due to the significantly greater number of slow myofibres per 'metabolic bundle' in the Large White pigs (Fig. 1); it was not due to a difference in the proportion of the deep region (see above) which was in fact greater in the miniature pigs. The superficial region of m. semitendinosus contained very few slow myofibres; between 0 and 2 per 'metabolic bundle' in Large White pigs and negligible numbers (usually 0, but occasionally 1) in the miniature pigs. The numbers involved in the superficial region were relatively insignificant but if included could only accentuate the difference in slow myofibre content between the two strains. The number of slow myofibres per 'metabolic bundle' in m. trapezius was also significantly greater in the Large White pigs. However, although there was no significant difference in this parameter between the two muscles studied in the Large White pigs, for the miniature pigs there were significantly ($P < 0{\cdot}001$) more slow myofibres per 'metabolic bundle' in m. trapezius than in m. semitendinosus.

DISCUSSION

It is well established for many animals, including the pig (Staun, 1972; Stickland & Goldspink, 1973) that myofibre number exhibits no change with postnatal growth. It was therefore felt justified to group the numerical data as shown in Table 1. The results show that it is the difference in myofibre number which is totally responsible

for the muscle size difference between the Large White and miniature pigs; this was also noted by Stickland & Goldspink (1978). Myofibre size differences do not account for any muscle size difference. In fact, quite the converse was found in that, at any given live weight, miniature pigs have muscle fibres of greater cross sectional area (Fig. 2). This appears to be due to the fact that they are older for a given live weight because, at any given age, there was no significant difference between the two breeds with respect to myofibre cross sectional area (Fig. 2, Table 2). This is a very interesting result in that it appears to reflect some of the results found in pigs of the same breed but of varying age at the same slaughter weight. Hegarty & Allen (1978) and Powell & Aberle (1981) showed that runt pigs have myofibres of greater cross sectional area than their large littermates when slaughtered at the same body weight. Handel (1984) attributed this difference to the disparate ages of the littermates at similar slaughter weights. It would seem that, in the present investigation also, for the two breeds of pigs used, muscle fibre cross sectional area is more indicative of the age of the animal than its live weight. It could be assumed that the runt pigs mentioned above are a result of inadequate prenatal nutritional levels. It would appear, therefore, that pigs of disparate birth weights, due to either nutritional *or* genetic (present investigation) effects exhibit postnatal increases in myofibre size which are more related to age than to live weight. This relationship is clearly dependent on optimum postnatal growing conditions as it is well known that several factors, such as nutritional levels (Joubert, 1956), may affect muscle fibre size. It is normally assumed that muscle fibre size relates to body weight and that any relationship with age is secondary, but these results indicate that, in some situations for certain periods of growth, muscle fibre size is related more to the age of an animal than to its live weight.

For the reasons mentioned earlier, it is possible to extrapolate from the results the pattern of prenatal muscle development in terms of primary and secondary myofibres. In nutritionally smaller pigs of the same litter it has been shown (Wigmore & Stickland, 1983) that the reduction in overall myofibre number in given muscles is due to a reduction in the number of secondaries forming around each primary myofibre. Although this difference has also been found between the two breeds of pigs used here, the most important factor responsible for the overall myofibre number difference is the number of primary myofibres, which each form their own 'metabolic bundles'. This difference in primary myofibre number is not found in the nutritional situation except in some very severely runted pigs (Handel, 1984).

It is known that many locomotory muscles (Pullen, 1977; Bodine *et al.* 1982) contain relatively more slow myofibres in their deeper aspects which thereby take on a more postural function. This division is found in m. semitendinosus of both the Large White and miniature pigs used here. However, it has been shown very clearly that the miniature pigs contain a relatively much reduced slow myofibre content in m. semitendinosus which is approximately one third of that found in Large White pigs. This difference is due mainly to a difference in the number of slow myofibres per 'metabolic bundle' between the two breeds. This again contrasts with the 'nutritional' situation in the large and small birth weight littermates which show no difference in the number of slow myofibres per 'metabolic bundle' at later ages (Handel, 1984). The number of fast myofibres is, however, reduced in these situations and this has also been shown in rats after experimentally induced malnutrition during pregnancy (Bedi *et al.* 1982). It should be mentioned here that, although the

conversion of secondary myofibres to slow contracting characteristics begins prenatally, there is also some postnatal conversion (Davies, 1972) but there does not appear to be any significant conversion during the period under investigation here. It was therefore felt justifiable to group the data in Table 3 as shown. However, it is possible that heavier miniature pigs (similar in weight to the older age-control Large Whites) may contain more slow myofibres, although the range did extend beyond the 8 kg suggested by Handel (1984) as being the weight by which most slow fibre conversion had occurred in the Large White pig. The results for m. trapezius indicate that this muscle is significantly slower than the deep portion of m. semitendinosus for miniature pigs but comparable for Large Whites. M. trapezius is known to have an important postural role in helping to support the scapula on the trunk. However, despite some differences between muscles, it can still be clearly seen that the m. trapezius reflects the significant difference seen in m. semitendinosus between the breeds with respect to slow myofibre content.

One of the aims of commercial pig production has been to select animals of large body weight. The aim in miniature pig production has clearly been the reverse and has been achieved in the Göttingen stock partly by the re-introduction of wild pigs (Haring *et al.* 1966). The results suggest that the selection for large body weight, associated with commercial pig production, has resulted in the selection of muscles with more metabolic bundles containing more, thinner muscle fibres when compared on an equal weight basis. Stickland (unpublished observation) has also noted that wild wart hogs have significantly larger muscle fibres than commercial Large White pigs when compared at the same live weight. The selection for larger body size has also resulted in relatively more slow muscle fibres in given muscles which may be necessary to support the increased weight of the animal.

Taken overall, the results of this investigation appear to show that genetically small animals develop fewer muscle fibres in their muscles by a different mechanism to that exhibited by animals which are smaller due to nutritional deprivation *in utero*. There are also functional consequences of these differences reflected in the histochemical properties of the constituent muscle fibres.

SUMMARY

M. semitendinosus and m. trapezius (portion) were removed from eight miniature pigs ranging from 21 to 160 days of age and eight age-control as well as eight weight-control commercial Large White pigs. Complete transverse frozen sections were obtained for each muscle sample and stained for various enzyme activities including myosin adenosine triphosphatase activity which enabled the identification of 'metabolic bundles'. This in turn enabled conclusions to be made about the prenatal development of the muscle in terms of primary and secondary myofibres. The Large White pigs contained 173 % more muscle fibres in m. semitendinosus than did the miniature pigs. Primary myofibre number was found to be about four times more important than secondary to primary myofibre ratios in determining myofibre number in the two breeds of pigs. Both primary myofibre number and secondary to primary myofibre ratios were, however, significantly greater in Large White than in miniature pigs. When the age- and weight-control Large Whites were compared with the miniature pigs it was found that at any given live weight the miniature pigs had thicker myofibres whereas at the same age there was no significant difference. The total area of m. semitendinosus occupied by slow myofibres was about three

times greater in the Large White pigs; the functional aspects of this are discussed. It was concluded that genetically smaller animals develop fewer muscle fibres in their muscles by a different mechanism to that exhibited by animals which are smaller due to nutritional deprivation *in utero*.

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REFERENCES

ASHMORE, C. R., ADDIS, P. B. & DOERR, L. (1973). Development of muscle fibers in the fetal pig. *Journal of Animal Science* **36**, 1088–1093.

AZIZ-ULLAH. (1974). Studies on muscle development with particular reference to the effects of protein malnutrition. Ph.D. thesis, University of Hull.

BEDI, K. S., BIRZGALIS, A. R., MAHON, M., SMART, S. & WAREHAM, A. C. (1982). Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and adult refed animals. *British Journal of Nutrition* **47**, 417–431.

BEERMANN, D. H., CASSENS, R. G. & HAUSMAN, G. J. (1978). A second look at fiber type differentiation in porcine skeletal muscle. *Journal of Animal Science* **46**, 125–132.

BODINE, S. C., RAY, R. R., MEADOWS, D. A., ZERNICKE, R. F., SACKS, R. D., FOURNIER, M. & EDGERTON, V. R. (1982). Architectural, histochemical and contractile characteristics of a unique biarticular muscle: the cat semitendinosus. *Journal of Neurophysiology* **48**, 192–201.

DAVIES, A. S. (1972). Postnatal changes in the histochemical fibre types of porcine skeletal muscle. *Journal of Anatomy* **113**, 213–240.

EVERITT, G. C. (1968). Prenatal development of uniparous animals with particular reference to the influence of maternal nutrition in sheep. In *Growth and Development of Mammals* (ed. G. A. Lodge and G. E. Lamming), pp. 131–157. London: Butterworths.

GUTH, L. R. & SAMAHA, F. J. (1970). Research note: Procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28**, 365–367.

HANDEL, S. E. (1984). Effects of low birthweight on postnatal development of skeletal muscle in the pig. Ph.D. thesis, University of Edinburgh.

HANRAHAN, J. P., HOOPER, A. C. & MCCARTHY, J. C. (1973). Effects of divergent selection for body weight on fibre number and diameter in two mouse muscles. *Animal Production* **16**, 7–16.

HARING, F., GRUHN, R., SMIDT, T. & SCHEVEN, B. (1966). Miniature swine development for laboratory purposes. In *Swine in Biomedical Research* (ed. L. K. Bustad & R. O. McClellan), pp. 789–796. Washington: Battelle Memorial Institute.

HEGARTY, P. V. J. & ALLEN, C. E. (1978). Effect of prenatal runting on the postnatal development of skeletal muscles in swine and rats. *Journal of Animal Science* **46**, 1634–1640.

JOUBERT, D. M. (1956). An analysis of factors influencing postnatal growth and development of the muscle fibre. *Journal of Agricultural Science* **47**, 59–102.

LUFF, A. R. & GOLDSPINK, H. (1967). Large and small muscles. *Life Sciences* **6**, 1821–1826.

NACHLAS, M. M., TSOU, K., DE SOUZA, E., CHENG, C. & SELIGMAN, A. M. (1957). Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *Journal of Histochemistry and Cytochemistry* **5**, 420–436.

POWELL, S. E. & ABERLE, E. (1981). Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. *Journal of Animal Science* **52**, 748–756.

PULLEN, A. H. (1977). The distribution and relative sizes of three histochemical fibre types in the rat tibialis anterior muscle. *Journal of Anatomy* **123**, 1–19.

ROBINSON, D. W. (1969). The cellular response of porcine skeletal muscle to prenatal and neonatal nutritional stress. *Growth* **33**, 231–240.

SMITH, J. H. (1963). Relation of body size to muscle cell size and number in the chicken. *Poultry Science* **42**, 283–290.

STAUN, H. (1972). The nutritional and genetic influence on number and size of muscle fibres and their response to carcass quality in pigs. *World Review of Animal Production* **8**/3.

STICKLAND, N. C. & GOLDSPINK, G. (1973). A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16**, 135–146.

SWATLAND, H. J. & CASSENS, R. G. (1973). Prenatal development, histochemistry and innervation of porcine muscle. *Journal of Animal Science* **36**, 343–354.

TAKEUCHI, T. (1956). Histochemical demonstration of phosphorylase. *Journal of Histochemistry and Cytochemistry* **4**, 84.

WIGMORE, P. M. C. & STICKLAND, N. C. (1983). Muscle development in large and small pig fetuses. *Journal of Anatomy* **137**, 235–245.

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MUSCLE CELLULARITY AND BIRTH WEIGHT

S. E. HANDEL†
Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH

AND

N. C. STICKLAND
Department of Anatomy, Royal Veterinary College, Royal College Street, London NW1 0TU

ABSTRACT

A study of the effects of low birth weight on muscle cellularity was performed on 48 pedigree Large White pigs selected, from a total of 17 litters, on the basis of their weight at birth. Where possible, the largest male (mean birth weight of 1544 g), smallest male (1135 g), and runt (776 g) littermates were chosen. Fresh frozen, whole mid belly, sections of *m. semitendinosus* and samples of *m. trapezius* from each animal were stained for the demonstration of acid pre-incubated myosin adenosine triphosphatase. The use of this stain demonstrated groups of positively stained, slow-contracting, myofibres which were each surrounded by a compliment of negatively stained, fast-contracting, fibres which together constituted 'metabolic bundles'. The positions of metabolic bundles are indicative of the presence of single primary myofibres in the foetal muscle, all the other myofibres in the metabolic bundles being derived from subsequently formed secondary fibres. Determination of total myofibre number and primary fibre number were made for *m. semitendinosus* together with an estimation of the secondary to primary fibre-number ratios for both this muscle and for *m. trapezius*. Low birth weight was associated with a permanently reduced total muscle fibre number, proportionately in the order of 0·19 ($P < 0·001$) between large and runt littermates. A reduced muscle fibre number was not always associated with low birth weight, but when this was the case it was generated through a reduced secondary to primary fibre-number ratio ($P < 0·01$). Primary fibre number was not significantly affected in low birth-weight pigs except in extreme cases of runting.

INTRODUCTION

THE muscle mass of an animal is determined mainly by the number and the size of its constituent myofibres. Luff and Goldspink (1970), Miller, Garwood and Judge (1975) and Hooper (1982) have all performed studies which suggest that the number of fibres in the muscle is the most prominent factor in limiting the ultimate size of the muscle. Fibre-number increases in muscles after birth depend on the state of maturity of the muscle at birth. The occurrence of hyperplasia after birth can be regarded as an extension of the embryonic differentiation of the muscle, as in the rat (Enesco and Leblond, 1962; Chiakulas and Pauly, 1965). Some animals, for example the mouse (Timson, 1982) and the pig (Staun, 1963; Stickland and Goldspink, 1973), apparently fail to show any increase in fibre number post natally.

Muscle fibres develop prenatally as two morphologically distinct populations. Fibres formed during the initial stages of myoblast fusion are primary myofibres which serve as a framework on which the smaller secondary myofibres develop (Ashmore, Robinson, Rattray and Doerr, 1972). In most porcine muscles, these two populations of myofibres can be differentiated from 90 days gestation by adenosine triphosphatase (ATPase) activity (Beerman, Cassens and Hausman, 1978). Animals of low birth weight tend to present reduced total myofibre counts (Bedi, Birzgalis, Mahon, Smart and Wareham, 1982; Wigmore and Stickland, 1983). Wigmore and

† Present address: Muscle Biology Laboratory, University of Wisconsin, Madison, WI 53706, USA.

Stickland (1983) suggested, after a study of prenatal porcine muscle development, that this was a result of a diminished secondary myofibre population, primary fibres remaining unaffected. In view of this hypothesis, an investigation into the effects of prenatal growth retardation, as exhibited by pigs of low birth weight, was performed on the post-natal cellularity of the muscle. Particular importance was placed on establishing the expression of the effects of total myofibre number with regards to the primary and secondary myofibre populations and whether the evident effects were of a permanent nature. Swatland and Cassens (1972) considered it possible that there could be a change in the apparent muscle fibre number after birth, the true number remaining fixed, due to the existence of intrafascicular terminations of fibres within the muscle. It was, therefore, important to consider this hypothesis with regards to the prenatally growth-retarded pig. Intrafascicular terminations of myofibres within the muscles of these animals, apparent as a reduced secondary myofibre population in the prenatal pig (Wigmore and Stickland, 1983), could grow and extend into the plane of sectioning (Swatland, 1976). This might be seen as an apparent increased myofibre number and, therefore, an extra parameter of muscle growth post natally. As mentioned above, muscle fibre number is the most important factor in limiting ultimate muscle size when comparing individual animals which have been reared under similar conditions. This present investigation therefore concentrates on muscle fibre number and its possible relationship with birth weight. Some of the results presented here have already appeared in a preliminary communication (Handel and Stickland, 1984).

MATERIAL AND METHODS

The *m. semitendinosus* and *m. trapezius* used for this investigation were dissected from the left side of 48 carcasses of pedigree Large White pigs. The pigs were selected from a total of 17 litters on the basis of their weight at birth. A maximum of three pigs were chosen from one litter; the largest male, the smallest (not less than 1000 g) male, and the runt (950 g or less). Small littermates were chosen to have birth weights just above that considered 'critical' (about 1000 g) by Hegarty and Allen (1978) and Powell and Aberle (1980), while the runt littermates were born of a weight below this critical weight. Although the sex of the pig does not appear to influence total muscle fibre number (Stickland, 1973), for the sake of consistency male pigs were selected where possible. However, over the period of this study the availability of male runts was limited and 59% of runts were gilts.

Representative littermates from the chosen litters were slaughtered, by exsanguination following electrical stunning, at various ages between birth and 128 days, littermates being slaughtered at the same age. After the dissection of the two muscles a complete mid-belly section (2 to 3 mm thick) was taken from *m. semitendinosus,* and from *m. trapezius*: a strip, up to 20 mm in length, was cut from mid way along the caudal edge of the thoracic portion. The muscle slices were then rapidly frozen in dichlorodifluoromethane (Arton 12, ICI Ltd) cooled to its melting point of −158°C with liquid nitrogen. Sections 10 μm in thickness, cut on a Slee retracting rotary cryostat at −22°C, were picked up on coverslips and allowed to thaw and dry out at room temperature for about 1·5 h.

Muscle sections were stained for the demonstration of ATPase following acid pre-incubation at pH 4·35 (Guth and Samaha, 1970). Acid pre-incubation was preferred to alkaline pre-incubation as it appeared to show greater fibre-type differentiation particularly in the neonatal samples. The number of acid ATPase-positive fibres in a muscle increases with post-natal development (Davies, 1972; Kugelberg, 1976). These fibres are characteristically grouped together within porcine muscle (Davies, 1972), an arrangement which reflects the original position of the first acid ATPase-positive fibres, i.e. the primary fibre population (Ashmore *et al.*, 1972; Ashmore, Addis and Doerr, 1973; Beermann *et al.*, 1978). Recording the number of groups of acid ATPase-positive fibres within a known area

followed by counting all remaining fibres within that area therefore enabled a quantification of the primary and secondary myofibre populations, respectively. This procedure was performed on five areas, each containing an average of 500 to 1500 fibres, randomly selected from sections of *m. trapezius* projected on to a screen of white card while areas from *m. semitendinosus,* containing a similar number of fibres, were projected to amount to at least 10 000 counted fibres. Evaluation of the secondary to primary fibre-number ratios was carried out for both muscles. In addition, *m. semitendinosus* provided total myofibre and primary fibre-number estimates for the whole muscle after determination of the total transverse sectional area of the muscle.

Regression analysis of the numbers of each fibre population against age and live weight were conducted to establish any post-natal changes. Paired observations were analysed to test the significance of the difference between mean values of the data from littermate groups (paired *t*-test).

RESULTS

Birth weights

Table 1 shows the birth weights, slaughter weights and ages of all the pigs used in this study. The mean birth weights exhibited by the large, small and runt littermate categories were 1544 (s.d. 189) g (no. = 17), 1135 (s.d. 191) g (no. = 12), and 776 (s.d. 107) g (no. = 19), respectively. The difference in birth weight between each group was highly significant ($P < 0{\cdot}001$). Of the 17 selected litters, five failed to present pigs definable as 'small' littermates and two litters contained two runt pigs which led to the unequal numbers within each littermate category (Table 1).

Fibre number

Regression analysis of the total number of myofibres in *m. semitendinosus* against live weight for each individual littermate category indicated that a constant number of myofibres was maintained in this muscle after birth in all animals, regardless of their weight at birth. The summated regression equation for

TABLE 1
Age and weight details of animals used

Age (days) at slaughter	Littermate category	Birth weight (g)	Live weight (g) at slaughter
0	Large	1 700	1 700
	Small	1 125	1 125
	Runt	900	900
1	Large	1 100	1 220
	Runt	900	1 000
	Runt	625	750
2	Large	1 800	2 075
	Small	1 000	1 200
	Runt	750	600
4	Large	1 800	2 200
	Small	1 100	1 700
	Runt	900	1 350
6	Large	1 450	2 150
	Small	1 000	1 500
	Runt	750	1 200
6	Large	1 350	2 450
	Runt	625	1 100
9	Large	1 800	4 500
	Small	1 700	3 100
	Runt	700	650
12	Large	1 500	4 225
	Runt	850	2 625
	Runt	750	2 500
15	Large	1 400	1 700
	Small	1 100	2 750
	Runt	600	675
19	Large	1 650	5 200
	Small	1 200	3 500
	Runt	950	2 900
27	Large	1 600	6 450
	Small	1 000	4 600
	Runt	800	3 400
33	Large	1 600	7 490
	Small	1 200	4 950
	Runt	650	4 350
46	Large	1 400	8 600
	Small	1 050	8 000
	Runt	750	5 000
64	Large	1 450	15 455
	Runt	750	8 410
84	Large	1 600	26 750
	Small	1 050	21 750
	Runt	900	28 250
100	Large	1 400	35 500
	Small	1 000	22 500
	Runt	750	31 000
128	Large	1 600	49 500
	Small	1 100	38 750
	Runt	900	40 000

m. semitendinosus total fibre number against live weight is $Y = -0{\cdot}349X + 417\,900$ where Y = fibre number and X = live weight (g) (the regression coefficient is not significantly different from 0). *M. semitendinosus* contained an average of 414 760 (s.d. 90 750) fibres for all animals studied.

The total myofibre number estimates for *m. semitendinosus* were significantly lower, proportionately by an average of 0·19, in the runts compared with their large siblings ($P < 0{\cdot}001$). Fibre-number differences between large and small, and small and runt littermates were not significant.

The low birth weights exhibited by subjects of the runt category were not consistently associated with reduced total fibre numbers. However, in cases where low birth weight was associated with a lowered total fibre number the relative importance of the primary and secondary fibre populations in contributing to this effect was investigated. The exclusion of pairs of siblings that failed to exhibit a disparity in total fibre number increased the average difference in total fibre number from proportionately 0·19 (no. = 19) to 0·25 (no. = 15).

The exclusion of two animals from the runt category was deemed necessary for some calculations as they appeared to constitute a sub-population suffering from severe prenatal growth retardation. These pigs had birth weights of more than 2·5 s.d. below their mean litter weight and were proportionately less than 0·50 of their respective large littermates. The exclusion of these two animals, despite possessing total fibre counts for *m. semitendinosus* which were proportionately only 0·38 and 0·49 of the values obtained for their respective large siblings, did not reduce the significance of the difference in fibre number between large and runt littermate groups (Table 2).

Primary fibre number

Regression analysis of primary fibre number against live weight and age demonstrated that the number of primaries in *m. semitendinosus* was independent of the age and live weight of the animal; the regression was not significant. The average number of primary fibres estimated for *m. semitendinosus*, from all animals, was 17 460 (s.d. 3 870).

Large and runt littermates exhibiting a difference in total *m. semitendinosus* fibre number also exhibited a significant difference in primary fibre number ($P < 0{\cdot}05$); runt littermates contained on average proportionately 0·15 fewer primary fibres (Table 2). However, by eliminating the two severely runted pigs from the analysis the effect of birth weight on primary fibre number lost its significance (Table 2). The extremely low primary fibre numbers of these two animals, proportionately 0·73 and 0·45 of the values estimated for their large littermates, were apparently responsible for the significance of the difference in primary fibre number seen between the large and runt littermates.

Secondary to primary fibre-number ratio

The lack of change demonstrated in the total myofibre and primary fibre numbers of *m. semitendinosus* after birth indicated that the secondary fibre population likewise remained constant. As estimates of total myofibre and primary fibre number were not available for *m. trapezius*, regression analysis of the secondary to primary fibre-number ratios of this muscle with live weight and with age was conducted and found not to exhibit any significant changes after birth, as deduced for *m. semitendinosus*. The secondary to primary fibre-number ratios of *m. semitendinosus* and *m. trapezius* were, on average, 23·9 (s.d. 3·3) and 22·0 (s.d. 3·2), respectively, when all three birth weight categories were considered.

Runting, in the instances where it was associated with a reduced total myofibre number in *m. semitendinosus*, significantly reduced the average secondary to primary fibre-number ratio by about 0·13 (Table 2), from 25·3 to 22·1 ($P < 0{\cdot}01$). Since the total fibre number of one muscle is indicative of that of other muscles in the body (Stickland and Goldspink, 1973), runting was assumed to affect the number of myofibres in *m. trapezius* only in cases where it affected *m. semitendinosus* fibre number. On investigation, the average secondary to primary fibre-number ratio of *m. trapezius* of these runts

TABLE 2

Mean proportional differences between paired observations from muscles of large and runt littermate groups

Parameter	Mean proportional difference: Large : runt (no. = 15)	Large : runt (excluding extreme runts) (no. = 13)
M. semitendinosus total fibre number	0·25***	0·21***
M. semitendinosus primary fibre number	0·15*	†
M. semitendinosus secondary to primary fibre-number ratio	0·13**	0·11**
M. trapezius secondary to primary fibre-number ratio	0·10**	0·06*

† Not significant ($P > 0·05$).

showed a 0·10 proportional reduction below that of the large littermates (Table 2) from 22·9 to 20·7 ($P < 0·01$). Exclusion of the two runts, which appeared to constitute a subpopulation, from data analysis failed to render the difference in secondary to primary fibre-number ratio between large and runt littermate categories insignificant although the proportional differences were reduced slightly to 0·11 ($P < 0·01$) and 0·06 ($P < 0·05$), for *m. semitendinosus* and *m. trapezius*, respectively.

DISCUSSION

The lack of change in the total fibre number of *m. semitendinosus* between birth and 128 days in all littermates supports the general concensus that muscle fibre number is fixed by birth in the pig (Staun, 1963; Stickland and Goldspink, 1973). In addition, this has been extended by the present work to include the prenatally growth-retarded pig, a case not previously studied as a possible deviation from the norm with regards to muscle fibre number. The present study rules out the possibility that runting delays the aquisition of the apparent mature fibre number until the post-natal period through, for example, limiting longitudinal growth of secondary muscle fibres prenatally and thus their presence in the muscle mid belly after birth, as the work of Swatland (1976) suggested. Therefore, the proportional overall fibre number reduction of 0·19 ($P < 0·001$), seen in light compared with heavy birth-weight pig littermates was maintained throughout the post-natal period despite adequate nutrition.

The degree of reduction in fibre number was comparable with that found by Wigmore and Stickland (1983) between the *m. semitendinosus* of large and small pig foetuses during the time of actual myofibre formation. The evident reduction in muscle fibre number of small foetal (Wigmore and Stickland, 1983) and post-natal pigs correlates with the effects seen on the muscle fibre number of experimentally undernourished foetal lambs (Everitt, 1968), and foetal and sucking rats (Bedi *et al.*, 1982). The considerable effect caused by prenatal growth retardation on muscle cellularity, as exemplified by the present study, strongly suggests, therefore, that the underlying cause is undernutrition, a concept further supported by the similarities in body composition, with respect to organ weight, evident between pigs of low birth weight (Widdowson, 1970, 1971 and 1974; Flecknell, Wootton and Royston, 1981; Wigmore, 1982) and human 'small-for-dates' babies that are considered to be the probable result of inadequate nutition (Naeye, 1965; Davies, Platts, Pritchard and Wilkinson, 1979).

In the present study, a significant difference was found to exist between the primary fibre numbers of the highest and lowest birth-weight littermates ($P < 0·05$) only with the inclusion of data from two exceptionally runted pigs. These two animals probably constituted a subpopulation (Royston, Flecknell and Wootton, 1982) of such severe growth retardation as to affect primary fibre formation. The phenomenon attributable to the two extreme runts, and presumably their severely affected muscle fibre numbers, was their respective birth weights relative to their siblings. Both runts were not only less than half the weight of their respective large littermates at birth but were also more than 2·5 s.d. below the mean litter weight, a combination which together accounted for their unique condition, i.e. a severely reduced muscle fibre number together with an affected primary myofibre

number and a lack of live-weight gain to slaughter at 9 and 15 days of age. It is therefore proposed that the stipulations stated above, which are more stringent than those previously put forward in the literature, constitute a 'critical' weight at birth, below which the individual's growth potential will be limited.

Within the normal range of growth retardation manifest within a litter of pigs, muscle fibre-number changes were apparently imposed only by effects on the secondary myofibre population, confirming the hypothesis put forward by Wigmore and Stickland (1983) after discovering a lower population of developing secondary muscle fibres in small prenatal pig foetuses. The results of the present study make it apparent that the prenatal growth restriction evident in low birth-weight pig littermates actually affects the formation of secondary myofibres. If the growth inhibition suffered by these pigs *in utero* had merely affected the longitudinal growth of the secondary population of muscle fibres, it is possible that, with adequate nutrition, they could grow and extend into the plane of muscle sectioning at the mid belly (Swatland and Cassens, 1972). This phenomenon would be seen as an increase in apparent muscle fibre number in the low birth-weight littermate group. However, the lack of a significant change in muscle fibre number in these pigs after birth suggests that, when reared under standard conditions of production, low birth-weight pigs have a permanently reduced muscle fibre number and, therefore, a reduced potential for meat production. This reduced potential has been shown in this study to be brought about by a reduction in the number of myofibres in each metabolic bundle whilst the total number of metabolic bundles in the muscle is not affected.

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REFERENCES

ASHMORE, C. R., ADDIS, P. B. and DOERR, L. 1973. Development of muscle fibers in the fetal pig. *Journal of Animal Science* **36:** 1088-1093.

ASHMORE, C. R., ROBINSON, D. W., RATTRAY, P. V. and DOERR, L. 1972. Biphasic development of muscle fibers in the fetal lamb. *Experimental Neurology* **37:** 241-255.

BEDI, K. S., BIRZGALIS, A. R., MAHON, M., SMART, J. L. and WAREHAM, A. C. 1982. Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and refed adult animals. *British Journal of Nutrition* **47:** 417-431.

BEERMANN, D. H., CASSENS, R. G. and HAUSMAN, G. J. 1978. A second look at fiber type differentiation in porcine skeletal muscle. *Journal of Animal Science* **46:** 125-132.

CHIAKULAS, J. J. and PAULY, J. E. 1965. A study of postnatal growth of skeletal muscle in the rat. *Anatomical Record* **152:** 55-61.

DAVIES, A. S. 1972. Postnatal changes in the histochemical fibre types of porcine skeletal muscle. *Journal of Anatomy* **113:** 213-240.

DAVIES, D. P., PLATTS, P., PRITCHARD, J. M. and WILKINSON, P. W. 1979. Nutritional status of light-for-date infants at birth and its influence on early postnatal growth. *Archives of Disease in Childhood* **54:** 703-706.

ENESCO, M. and LEBLOND, C. P. 1962. Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. *Journal of Embryology and Experimental Morphology* **10:** 530-562.

EVERITT, G. C. 1968. Prenatal development in uniparous animals with particular reference to the influence of maternal nutrition in sheep. In *Growth and Development of Mammals* (ed. G. A. Lodge and G. E. Lamming), pp. 131-157. Butterworths, London.

FLECKNELL, P. A., WOOTTON, R. and ROYSTON, J. P. 1981. Pathological features of intrauterine growth retardation in the piglet: differential effects on organ weights. *Diagnostic Histopathology* **4:** 295-298.

GUTH, L. and SAMAHA, F. J. 1970. Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28:** 365-367.

HANDEL, S. E. and STICKLAND, N. C. 1984. Muscle cellularity and its relationship with birthweight and growth. *Journal of Anatomy* **136:** 726 (Abstr.).

HEGARTY, P. V. J. and ALLEN, C. E. 1978. Effect of pre-natal runting on the post-natal development of skeletal muscle in swine and rats. *Journal of Animal Science* **46:** 1634-1640.

HOOPER, A. C. B. 1982. Genetic influences on muscle growth. *Journal of Muscle Research and Cell Motility* **3:** 113 (Abstr.).

KUGELBERG, E. 1976. Adaptive transformation of rat soleus motor units during growth. Histochemistry and contraction speed. *Journal of the Neurological Sciences* **27:** 269-289.

LUFF, A. R. and GOLDSPINK, G. 1970. Total number of fibers in muscles of several strains of mice. *Journal of Animal Science* **30:** 891-893.

MILLER, L. R., GARWOOD, V. A. and JUDGE, M. D. 1975. Factors affecting porcine muscle fibre type, diameter and number. *Journal of Animal Science* **41:** 66-77.

NAEYE, R. L. 1965. Probable cause of foetal growth retardation. *Archives of Pathology* **79:** 284-291.

POWELL, S. E. and ABERLE, E. D. 1980. Effects of birth weight on growth and carcass composition of swine. *Journal of Animal Science* **50:** 860-868.

ROYSTON, J. P., FLECKNELL, P. A. and WOOTTON, R. 1982. New evidence that the intrauterine growth retarded piglet is a member of a discrete subpopulation. *Biology of the Neonate* **42:** 100-104.

STAUN, H. 1963. Various factors affecting number and size of muscle fibres in the pig. *Acta Agriculturae Scandinavica* **13:** 293-322.

STICKLAND, N. C. 1973. The growth and development of skeletal muscle in pigs. *Ph.D. Thesis, Univ. Hull.*

STICKLAND, N. C. and GOLDSPINK, G. 1973. A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16:** 135-146.

SWATLAND, H. J. 1976. Effect of growth and plane of nutrition on apparent muscle fibre numbers in the pig. *Growth* **40:** 285-292.

SWATLAND, H. J. and CASSENS, R. G. 1972. Muscle growth: the problem of muscle fibers with an intrafascicular termination. *Journal of Animal Science* **35:** 336-344.

TIMSON, B. F. 1982. The effect of varying postnatal growth rate on skeletal muscle fibre number in the mouse. *Growth* **46:** 36-45.

WIDDOWSON, E. M. 1970. Harmony of growth. *Lancet* **1:** 901-905.

WIDDOWSON, E. M. 1971. Intra-uterine growth retardation in the pig. 1. Organ size and cellular development at birth and after to maturity. *Biology of the Neonate* **19:** 329-340.

WIDDOWSON, E. M. 1974. Immediate and long-term consequences of being large or small at birth. In *Size at Birth* (ed. K. Elliot and J. Knight), pp. 65-82. Elsevier, Amsterdam.

WIGMORE, P. M. C. 1982. Prenatal muscle development in the pig. *Ph.D. Thesis, Univ. Edinburgh.*

WIGMORE, P. M. C. and STICKLAND, N. C. 1983. Muscle development in large and small pig fetuses. *Journal of Anatomy* **32:** 235-245.

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Relationship between Motoneuron Number and Myofibre Number in the Pig

N.C. Stickland

Department of Anatomy, The Royal Veterinary College, London, UK

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Abstract. A segment (at level T1) of spinal cord and a midbelly slice of the m. semitendinosus were removed from the largest- and smallest-birthweight littermate of each of 12 Large White pig litters sacrificed at various ages. When the ratio of myofibre numbers for large to small littermate within each litter was related to the ratio of motoneuron numbers then a significant correlation ($r = 0.638$, $p < 0.05$) was observed. It is suggested that in utero growth retardation causes a reduction in myofibre numbers (within a litter), which in turn reduces motoneuron survival rate.

Introduction

Loss of motoneurons during development has been observed in all animals which have been studied, including amphibians [Hughes, 1961], chicks [Hamburger, 1975] and mammals [Harris-Flannagan, 1969]. Amputation of limb buds reduces the survival of motoneurons in the spinal cord segments supplying the limb; this reduction is proportional to the amount of limb removed [Lamb, 1981]. Conversely, supernumerary limbs provide an increased field of innervation so that motoneuron numbers are increased in the spinal cord on the side with the extra limb [Hollyday and Hamburger, 1976; Lamb, 1979]. The results of these experiments indicate that motoneuron survival is proportional to the size of the field of innervation.

In an investigation on porcine muscle [Handel, 1984] it was found that within one litter the variation in muscle fibre numbers in given muscles was up to 170% when comparing the largest and smallest littermates. It was decided to investigate whether this naturally occurring large variation in myofibre number was related to any variation in motoneuron number.

Materials and Methods

The largest- and smallest-birthweight pigs were selected from each of 12 Large White pig litters. Each pair of animals was then sacrificed at ages ranging from 6 to 97 days. The m. semitendinosus was removed from the left side of each animal and 10 μm frozen transverse sections were obtained across the complete muscle at its mid-length position. The m. semitendinosus was used as it is known that the myofibre number in this muscle in the pig does not change with age [Handel and Stickland, in press]. The sections were stained with haematoxylin and eosin and the numbers of muscle fibres observed in randomly selected areas of known size were noted. In this way 2–3% of the total number were counted in order to obtain a figure for the number of muscle fibres per unit area. This figure was used with the measured total cross-sectional area of the muscle to calculate the total number of muscle fibres in the complete section.

A segment (at level T1) of spinal cord was also removed from each pig and fixed in buffered 10% formol saline. More caudal spinal segments could not be used in this investigation as the caudal halves of the animals were used in another investigation. However, it is known that within the same pig, fibre numbers in one muscle are closely related to fibre numbers in other muscles [Stickland and Goldspink, 1973]. Therefore fibre numbers in muscles supplied by T1 motoneurons would relate to those in the semitendinous muscle. The spinal cord segment was processed, embedded in wax and 10 μm transverse sections were cut from the middle third of the segment. The sections were stained in 1% toluidine blue and the number of motoneurons exhibiting nucleoli [Konigsmark, 1970] were

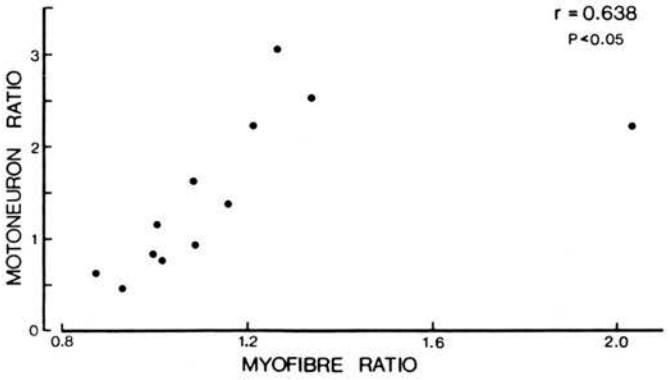

Fig. 1. Relationship between 'motoneuron number index' ratio (between largest and smallest littermate) and a similar ratio for total myofibre number in m. semitendinosus. Each point represents one litter.

Table I. Motoneuron number index and total myofibre number in m. semitendinosus for all large and small littermate pigs used

Age, days	Motoneuron index		Myofibre number (× 1,000)	
	large	small	large	small
6	800	495	419	300
6	425	304	501	432
12	840	332	479	357
15	777	347	263	129
27	985	1,269	348	341
33	352	115	491	388
46	1,288	1,109	414	409
64	671	301	426	350
69	288	624	571	608
84	1,365	1,641	433	433
85	544	588	483	443
97	423	676	474	540

counted in the ventral gray matter (ventral to a horizontal line through the central canal) of one side in every 10th section until 10 sections had been counted. Owing to the different size of the animals used a 'motoneuron number index' was obtained by multiplying the average number per section by the crown-rump length (in cm) of the animal concerned.

Results

Table I lists the animals used in this investigation together with the motoneuron and myofibre number results obtained.

There was no evidence (by regression analysis) in this investigation of any change in either myofibre number or motoneuron number with age. However, it can be seen that the variation in motoneuron number between litters is considerable. Much of the variation seen may be due to the different ages of the litters used. Age-related factors which may affect the results include postnatal neuron death, difficulty in distinguishing immature neurons and changes in the distribution of motoneuron pools. It was decided therefore to concentrate on within-litter variation so that these age-related factors could be excluded. In figure 1 the motoneuron number indices and myofibre numbers are each expressed as ratios of large to small littermates. It is difficult to explain the isolated litter (with a myofibre ratio of 2.04) seen in figure 1; it can only be said that the two pigs in this litter contained the two lowest myofibre number values encountered in this investigation (see table I). The maximum difference within litters for myofibre numbers was 104% and for motoneuron numbers it was 206%; the mean difference for these parameters was 17 and 48%, respectively.

Discussion

The most interesting observation to have emerged from this investigation is that within litters of pigs there does appear, from figure 1, to be a fairly close relationship between motoneuron number and myofibre number; the littermate with more myofibres in its muscle has more motoneurons than its sibling. This present investigation appears therefore to support the findings of other work using experimental techniques which have indicated that motoneuron survival is proportional to the size of the field of innervation [Lamb, 1979, 1981; Hollyday and Hamburger, 1976]. As mentioned above, although the myofibre numbers used are for m. semitendinosus, Stickland and Goldspink [1973] showed that myofibre number in one muscle is related to that in another muscle in the same animal. In other words, it is reasonable to suppose that myofibre numbers in muscles supplied by motoneurons in segment T1 (used here) would relate to the numbers in m. semitendinosus.

The mean difference of 17% in myofibre numbers in m. semitendinosus between large and small littermates is of the same order as that observed by Wigmore and Stickland [1983] in a study of prenatal muscle development in the pig. These authors concluded that the smallest littermate was small as a result of its disadvantaged site within the uterus which caused it to receive inadequate nutrition.

A suggested mechanism whereby the reduced myofibre number is produced in these pigs was put forward by these authors.

It is known that food restriction during pregnancy also causes a reduced cell number in nervous tissue including the spinal cord [Schrader and Zeman, 1969]. It is possible that this is an indirect effect due to the food restriction bringing about a reduction in myofibre number, i.e., a smaller field of innervation, so that fewer motoneurons survive.

It may be concluded that, within a litter, in utero-growth-retarded animals develop fewer myofibres in their muscles which may result in (or which at least is related to) a reduction in motoneuron survival. More work, involving horseradish peroxidase labelling of specific motoneuron pools, is now required to adequately test this hypothesis which is suggested from the results available in this study.

Acknowledgement

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References

Hamburger, V.: Cell death in the development of the lateral motor column of chick embryo. J. comp. Neurol. *160:* 535–546 (1975).

Handel, S.E.: Effects of low birthweight on postnatal development of skeletal muscle in the pig; Ph.D. thesis University of Edinburgh (1984).

Handel, S.E.; Stickland, N.C.: Muscle cellularity and birthweight. Anim. Prod. (in press).

Harris-Flannagan, A.E.: Differentiation and degeneration in the motor horn of the foetal mouse. J. Morph. *129:* 281–306 (1969).

Hollyday, M.; Hamburger, V.: Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. J. comp. Neurol. *170:* 311–320 (1976).

Hughes, A.: Cell degeneration in the larval ventral horn of *Xenopus laevis* (Daudin). J. Embryol. exp. Morph. *9:* 269–284 (1961).

Konigsmark, B.W.: Methods for the counting of neurons; in Nauta, Ebberson, Contemporary research methods in neuroanatomy, pp. 315–340 (Springer, New York 1970).

Lamb, A.H.: Ventral horn cell counts in a *Xenopus* with naturally occurring supernumerary hindlimbs. J. Embryol. exp. Morph. *49:* 13–16 (1979).

Lamb, A.H.: Target dependency of developing motoneurons in *Xenopus laevis.* J. comp. Neurol. *203:* 157–171 (1981).

Shrader, R.E.; Zeman, F.J.: Effect of maternal protein deprivation on morphological and enzymatic development of neonatal rat tissue. J. Nutr. *99:* 401–421 (1969).

Stickland, N.C.; Goldspink, G.: A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. Anim. Prod. *16:* 135–146 (1973).

Wigmore, P.M.C.; Stickland, N.C.: Muscle development in large and small pig fetuses. J. Anat. *137:* 235–245 (1983).

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N.C. Stickland,
Department of Anatomy,
The Royal Veterinary College,
Royal College Street,
UK–London NW1 OTU (United Kingdom)

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The growth and differentiation of porcine skeletal muscle fibre types and the influence of birthweight

S. E. HANDEL AND N. C. STICKLAND

Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh, Scotland and * *Department of Anatomy, The Royal Veterinary College, Royal College Street, London NW1 0TU, England*

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INTRODUCTION

Skeletal muscle is composed of a heterogeneous population of myofibres that differ in physiological (Burke *et al.* 1971) and biochemical (Sréter, Seidel & Gergely, 1966) properties. A myofibre's indigenous physiological and metabolic properties contribute to the effectiveness and efficiency of the functioning of the skeletal muscle with respect to the support and movement of the body. The most comprehensive system of nomenclature for myofibre 'typing' gives a full description of these physiological and metabolic capacities, based on the relative contraction speed of the fibre and its propensity for oxidative and glycolytic metabolism (Peter *et al.* 1972). Accordingly, these workers described three basic myofibre types in adult guinea-pig and rabbit limb muscles that are common to most mammalian muscles: (i) slow-twitch, oxidative metabolism (SO), (ii) fast-twitch, oxidative and glycolytic metabolism (FOG), and (iii) fast-twitch, glycolytic metabolism (FG). The histochemical staining patterns of myofibres are indicative of their physiological and biochemical properties.

Changes in the proportions of fast- and slow-contracting fibres occur naturally in the growing animal after birth. The growth process is associated with an increasing functional load due to liveweight gain and the way in which the muscle maintains a supportive role appears to be through an increase in the percentage of slow-contracting, fatigue-resistant fibres (Edström & Kugelberg, 1968; Davies, 1972). With growth the number of slow fibres in the muscle is augmented through the conversion of fast fibres since total muscle fibre number remains constant postnatally (Staun, 1963; Stickland & Goldspink, 1973).

The progenitors of the initial population of slow-contracting (acid-adenosine triphosphatase-stable) fibres in prenatal muscle are called primary fibres (Ashmore, Robinson, Rattray & Doerr, 1972*b*; Ashmore, Addis & Doerr, 1973). These fibres, once formed, act as a framework on which myoblasts align and fuse to form a population of secondary fibres. A proportion of the fast-contracting population of secondary fibres are the progenitors of slow fibres postnatally in mixed fibre and predominantly slow fibre type muscles. The lower ($P < 0{\cdot}001$) total muscle fibre number in low birthweight, runt pigs compared to their heavy birthweight littermates has been mainly attributed to a reduced secondary fibre population (Handel & Stickland, 1984). Low birthweight pigs also appear to suffer a consistently reduced postnatal liveweight relative to their heaviest birthweight siblings (Hegarty & Allen,

* Present address of both authors.

Table 1. *Classification of myofibre types*

Histochemical staining pattern				Myofibre type classification
Acid ATPase	Alkaline ATPase	SDHase	GPase	
+	−	+	−	SO
+	/	+	−	IOa
+	+	+	−	IOb
/	/	+	−	IOc
/	+	+	−	IOd
−	/	+	−	FO
−	+	+	−	FO
−	/	+	+	FOG
−	+	−	+	FG

+, Positive staining reaction; /, intermediate staining reaction; −, negative staining reaction.

1978; Powell & Aberle, 1981). It was therefore considered valuable to investigate the effects that these phenomena might impose on the proportions and growth rates of the various muscle fibre types between littermates, and as a consequence the comparative physiological and biochemical properties of their muscles. In addition, a comprehensive study of this kind in pigs of disparate birthweight, and subsequently disparate liveweight gain, would enable the comparison of the muscle development of pigs of the same age (but different liveweights) or of equal liveweight (but different ages). This would therefore help to elucidate the separate effects that both age and liveweight might impose on postnatal muscle growth and development.

MATERIALS AND METHODS

Muscle samples

Muscle samples taken for this study were from 49 pigs, from a total of 17 litters of a Large White herd. Selection of littermates was by weight at birth. Where possible 3 animals were chosen from each litter; the largest male, smallest (not less than 1000 g) normal male, and the runt (950 g or less). The ages at which the litters were slaughtered were 0, 1, 2, 4, 6, 9, 12, 15, 19, 27, 33, 46, 64, 84, 100 and 128 days. Each pig was weighed and then slaughtered by exsanguination after electrical stunning. The two muscles chosen for investigation, the semitendinosus and the trapezius, were dissected free immediately after slaughter from the left side of the carcass. A complete midbelly transverse section was then taken from the semitendinosus while from the trapezius a strip, up to 2 cm in length, was cut from midway along the caudal edge of the thoracic portion. Serial sections of 10 μm thickness were cut at −22 °C. Four histochemical techniques were applied to the serial sections from each muscle sample. The techniques used were: the demonstration of (i) acid and (ii) alkaline stable adenosine triphosphatase (acid and alkaline ATPase) as outlined by Guth & Samaha (1970), (iii) the demonstration of succinate dehydrogenase (Nachlas *et al.* 1957) and (iv) the demonstration of glycogen phosphorylase as described by Takeuchi (1956; a modification of the method outlined by Takeuchi & Kuriaki, 1955).

Table 2. *Myofibre type percentages in 'mature' muscle of pigs* (n = 11) *over a liveweight of* 8·5 *kg*

	Mean % (S.D.)		
	Semitendinosus		
Fibre type	'Superficial'	'Deep'	Trapezius
SO	3·0 (2·6)	38·5 (6·0)	40·4 (4·6)
IO	—	1·0 (1·1)	1·0 (1·1)
FO	—	2·7 (2·7)	2·7 (1·4)
FOG	26·9 (7·1)	35·7 (4·1)	23·5 (6·6)
FG	71·3 (7·8)	23·8 (5·8)	32·4 (9·4)

Procedure of myofibre typing

A range of intensity was apparent in the acid and alkaline ATPase staining of muscle sections from young animals. Therefore, fibres were classified according to whether they showed a positive, negative or intermediate reaction for acid and alkaline ATPase. In addition, fibres were classified as succinate dehydrogenase-positive or negative. Although a continuous spectrum of activity of this enzyme ranging from weak to strong was apparent, fibres possessing weak activity were recorded as non-oxidative. Finally, fibres were recorded as either glycogen phosphorylase-positive, if staining was detectable, or negative, if staining was negligible. A total of 9 different combinations of histochemical staining patterns was recorded and categorised under 5 major titles (SO, transitional types IO and FO, FOG and FG), as displayed in Table 1. The histochemical (ATPase) fibre type staining pattern of the superficial portion of the semitendinosus (Table 2) was distinct from that of the deep portion as it possessed an almost negligible number of slow fibres and was therefore characterised as a 'fast' muscle area. The deep portion of the semitendinosus and the whole of the trapezius presented a mixed myofibre type composition (Table 2). Therefore, three different muscle type areas or 'sections' were obtained from the two muscles studied. Myofibre type percentages were estimated from two randomly selected fascicles from sites within each of these three sections of muscle. This amounted to an average of approximately 600 fibres classified for each section of muscle from each littermate. The two randomly selected muscle fascicles from each muscle section stained for alkaline ATPase were projected onto white card. Each myofibre within the chosen fascicle was outlined and the staining intensity for each of the four histochemical techniques recorded. The number of myofibre types from the two selected fascicles were then summated to determine percentages of each myofibre type for each of the three muscle sections. This procedure was performed for each of the 49 pigs in this study.

Porcine mixed muscle has a unique arrangement of fibres in that the slow (alkaline ATPase-labile, acid ATP-stable; Table 1) fibres are grouped together in bundles. These groups of slow fibres and their surrounding complement of fast fibres are termed 'metabolic bundles' (Fig. 1) in the present report. The numbers of alkaline ATPase-negative (slow, SO) fibres associated with a known number of (at least 150) metabolic bundles were counted to obtain an average value for the muscles showing mixed fibre types, i.e. the deep portion of the semitendinosus and the trapezius, from each animal.

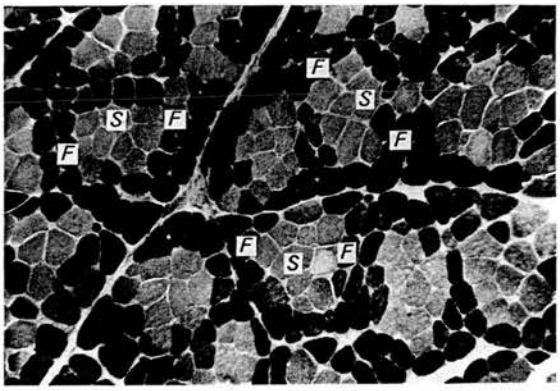

Fig. 1. Muscle fibres from the deep portion of the semitendinosus muscle of an 8·4 kg Large White pig stained for the demonstration of alkaline ATPase. Groups of slow-contracting, alkaline ATPase-positive (*S*) fibres and their surrounding complements of fast-contracting (*F*) fibres are termed 'metabolic bundles'. × 163.

Determination of the mean transverse sectional area of each myofibre type

The mean transverse sectional area (TSA) of 50 randomly selected fibres from each of three types classified as either SO, FOG (including FO) or FG were determined for the three muscle sections of each pig. TSA measurements were made of outlined fibres on a Reichert–Jung Videoplan. An estimate of the total TSA of slow muscle within the midbelly of the semitendinosus muscle from each littermate was made by multiplying the mean TSA values for slow fibres determined for both the deep and superficial portions of this muscle by the number of slow fibres estimated for each respective portion.

Analysis of data

The relative change in the histochemical properties and mean myofibre TSAs had to be quantified for a comprehensive analysis and a valid comparison between littermates. Regression equations were established for relationships between the measured parameters (i.e. slow fibres per metabolic bundle, proportions and TSAs of myofibre types) and both age and liveweight. The establishment of linear relationships between the TSAs of myofibre types and age or liveweight required logarithmic transformation of the X and Y variates. The resultant data enabled comparisons of changes in the number of slow fibres per metabolic bundle, and of changes in myofibre type percentages and TSA growth between littermate groups and also the analysis of differences in the TSA growth between fibre types. When two regression equations were established as having essentially equal coefficients, statistical comparison of their intercepts (Quenouille, 1969) enabled differences in their relative values of Y to be estimated for a given value of X; thus, when littermate groups showed similar rates of change with liveweight or age in any of the measured parameters, relative differences in the measured parameters between littermates of the same age or liveweight could be established.

RESULTS

Birthweights and liveweight gain

The mean birthweights ($\pm$S.D.) of the large, small and runt littermates were 1544 g $\pm$ 189 (n = 17), 1144 g $\pm$ 190 (n = 13) and 776 g $\pm$ 107 (n = 19), respectively.

The runt was, on average, about half the weight of the large littermate at birth, and the small littermate represented the median birthweight between that of the large and that of the runt. The difference in birthweight, calculated by a paired '*t*'-test, between each littermate category was highly significant ($P < 0{\cdot}001$). Liveweight differences, evaluated by regression analysis, were maintained between large and runt ($P < 0{\cdot}001$), and large and small ($P < 0{\cdot}01$) littermates throughout the period studied.

Myofibre type differentiation

In the neonatal pig all the muscle fibres of the semitendinosus and the trapezius exhibited positive succinate dehydrogenase staining. Differential staining began at 6 days with a reduction in the intensity of staining in the superficial portion of the semitendinosus. This differential staining appeared to be specific to a liveweight of approximately 2·5 kg in the superficial portion of the semitendinosus while in its deep portion and in the trapezius differential staining was not apparent until the heavier liveweight of 8·5 kg was attained (these liveweights corresponded to the ranges in age of 6 to 19 days and 33 to 64 days, respectively). Once differentiation with respect to succinate dehydrogenase had occurred there was no significant change in the proportion of oxidative fibres.

At birth the glycogen phosphorylase activity in all myofibres was negligible. Activity of this enzyme was detected at a liveweight of 2·5 kg (6 to 19 days) in all muscle sections. The proportion of glycolytic fibres remained constant after the liveweight reached 8·5 kg. Glycogen phosphorylase activity in the myofibres of the superficial portion of the semitendinosus coincided with the loss of 100% succinate dehydrogenase staining. Therefore, fibres classified as FOG and FG could be identified in the superficial portion of the semitendinosus (a predominantly fast muscle) after the attainment of 2·5 kg liveweight, whereas in the deep portion of this muscle and in the trapezius (examples of mixed muscles), although FOG fibres appeared at this liveweight, FG fibres were not observed until about 8·5 kg.

Neonatal pigs presented poor alkaline ATPase differential staining in their muscles. However, one slow fibre per metabolic bundle was apparent in the deep portion of the semitendinosus in each littermate just after birth, while the trapezius possessed an average of $2{\cdot}4 \pm 0{\cdot}2$ slow fibres per metabolic bundle. On average, $3{\cdot}7 \pm 0{\cdot}6$ and $4{\cdot}5 \pm 0{\cdot}6$ (mean ± S.D.) fibres per metabolic bundle were stained positively for acid ATPase just after birth in the deep portion of the semitendinosus and in the trapezius, respectively. The disparity in the number of acid ATPase-positive and alkaline ATPase-negative fibres in the young pig indicated the presence of a population of transitional fibres (Table 1).

Transitional myofibres

In mixed muscle from the deep portion of the semitendinosus and from the trapezius a gradation of myofibre types was shown to exist between the mature SO and FOG fibres. As evident from Table 1, six of these so called 'transitional' fibre types were identified and allocated to two classes, IO and FO. All transitional fibres were oxidative and non-glycolytic, and possessed ATPase activities that ranged in staining intensity from alkaline ATPase-high and acid ATPase-low, to alkaline ATPase-intermediate and acid ATPase-high. Those fibres classified as FO were considered transitional fibres only after glycogen phosphorylase staining had been

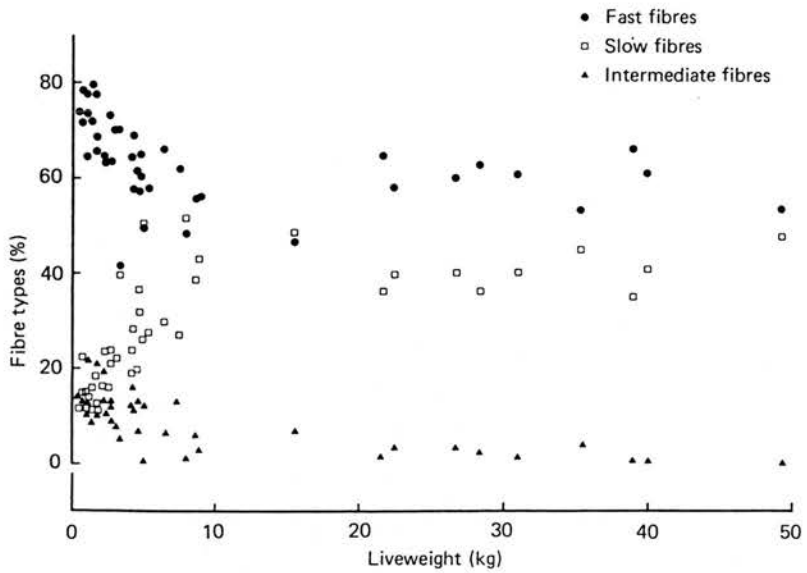

Fig. 2. Percentages of fast, slow and intermediate fibres (as classified in Table 1) in the trapezius muscle against liveweight.

detected in muscle sections; prior to this the majority were considered to be merely undifferentiated fibres and progenitors of the FOG fibre population.

The decrease in the percentage of transitional fibres with increasing liveweight appeared to be related to the attainment of the mature myofibre type ratios, especially those of the SO fibres. This is demonstrated by the myofibre type changes of the trapezius shown in Figure 2, which were similar to those seen within the deep portion of the semitendinosus. The negligible number of transitional fibres demonstrated in muscle from pigs heavier than 8·5 kg coincided with the constancy of the fibre type percentages that were considered representative of mature muscle (Table 2).

Changes in the proportions of myofibre types with growth

After birth the number of slow fibres per metabolic bundle, and therefore the percentage of slow fibres, in the deep portion of the semitendinosus and in the trapezius increased until a liveweight of approximately 8·5 kg was attained. Thereafter the number of slow fibres per metabolic bundle, and hence the percentages of slow fibres, were not shown to change significantly (Fig. 2). The superficial portion of the semitendinosus failed to show any slow fibres until 15 days postnatally but once slow fibres were established, their proportion remained constant. The percentages of slow fibres in the mature semitendinosus and trapezius muscles are given in Table 2. No significant differences existed between littermates of equal liveweight or age in the number of slow fibres per metabolic bundle of either the deep portion of the semitendinosus or of the trapezius. Likewise, the total percentages of slow fibres were not found to be significantly different between littermates of equal age in either of these muscles. However, on an equal liveweight basis the percentage of slow fibres in the trapezius was significantly greater ($P < 0{\cdot}05$) in the runt than the small littermate, while in the deep portion of the semitendinosus differences existed between the large and the runt ($P < 0{\cdot}001$) and the large and the small ($P < 0{\cdot}05$) littermate groups.

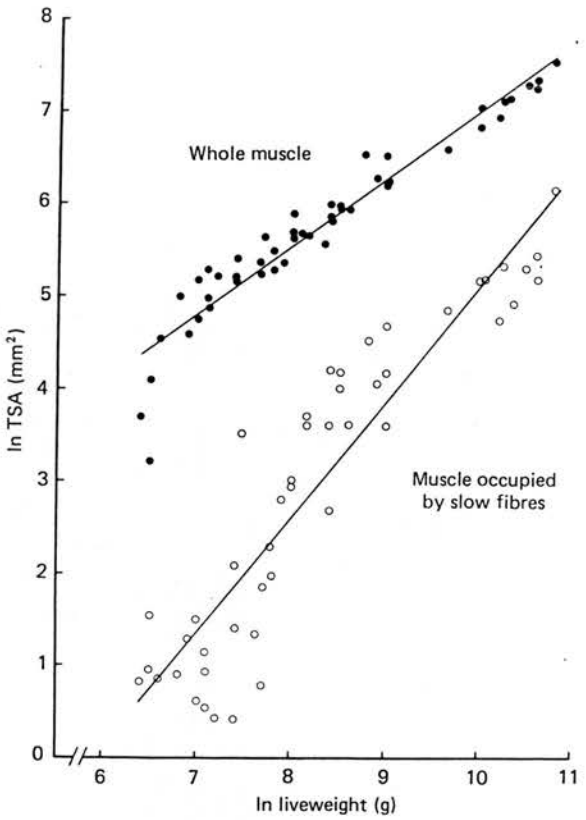

Fig. 3. Changes in the whole TSA of the semitendinosus muscle and the area of the muscle occupied by slow fibres with liveweight. Regression equations: Whole muscle, ln $Y = 0{\cdot}74$. ln $X - 0{\cdot}39$. Muscle occupied by slow fibres, ln $Y = 1{\cdot}23$. ln $X - 7{\cdot}26$.

Changes in the TSA of muscle occupied by slow myofibres

As shown in Figure 3, from birth to 128 days of age the TSA of the semitendinosus and the area occupied by slow fibres in the muscle increased with liveweight. The relationships established between the natural logarithms (ln) of these two parameters against both ln (liveweight) and ln (age) were essentially equal between littermates. The regression coefficient of the relationship between ln (semitendinosus TSA) and ln (liveweight) (Fig. 3) was not significantly different from 2/3, while the regression coefficient of ln (total area of slow muscle) and ln (liveweight) (Fig. 3) was significantly greater than 1·0 ($P < 0{\cdot}01$) and was best estimated at 5/4.

No significant difference existed in the area of slow muscle between littermates of the same age. However, the runt and small littermates both possessed a significantly ($P < 0{\cdot}001$) greater area of slow semitendinosus muscle than their respective large littermates on an equal liveweight basis.

Changes in the mean TSAs of myofibre types with growth and differences between littermates.

The regression coefficients of ln (myofibre mean TSAs) against both ln (age) and ln (liveweight) were not significantly different between littermate groups. This indicated that the rate of growth of myofibre TSAs was the same between litter-

Table 3. *Regression data for ln (myofibre type mean TSAs) against ln (liveweight)*

Muscle	Myofibre type	Regression coeffiients b (S.E.)†	Intercepts (ln) of regression lines			Significance of difference between intercepts		
			Large	Small	Runt	Large: Runt	Large: Small	Small: Runt
M ST*	SO	0·62 (0·04)	0·25	1·09	1·92	$P < 0·001$	$P < 0·001$	$P < 0·001$
Deep	FO+FOG	0·82 (0·04)	−0·87	0·34	−0·77	$P < 0·05$	$P < 0·001$	$P < 0·001$
Portion	FG	0·89 (0·04)	−1·52	−0·21	−1·26	NS	$P < 0·001$	$P < 0·05$
M ST*	SO	0·58 (0·05)	0·82	1·89	1·86	$P < 0·001$	NS	NS
Superf.	FO+FOG	0·95 (0·03)	−2·30	−1·94	−1·87	NS	$P < 0·01$	$P < 0·001$
Portion	FG	0·97 (0·05)	−2·25	−1·33	−1·97	$P < 0·001$	$P < 0·001$	NS
Trapezius	SO	0·67 (0·03)	0·18	0·03	0·58	$P < 0·01$	NS	$P < 0·001$
	FO+FOG	0·69 (0·03)	−0·37	0·79	−0·10	NS	$P < 0·001$	$P < 0·001$
	FG	0·76 (0·04)	−0·85	−0·37	−0·35	$P < 0·05$	$P < 0·001$	$P < 0·05$

*, Semitendinosus muscle. †, For large, small and runt TSAs combined.

mates. Comparisons of the intercepts of the regression lines for the natural logarithms of the various myofibre type mean TSAs and ln (age) for littermate groups revealed that littermates of the same age possessed myofibres of essentially equal TSAs. However, when a similar evaluation was made of specific myofibre type mean TSAs between littermates of the same liveweight they were not always found to be equal (see Table 3). Where significant differences existed, the myofibre types under consideration were larger in the lower birthweight littermate groups. It is interesting to note that the SO fibre mean TSAs in the deep portion of the semitendinosus were more significantly different ($P < 0·001$) between each littermate group than any other fibre type studied.

Regression coefficients for ln (myofibre mean TSAs) against ln (liveweight) for the SO myofibre types of the three muscle sections (as defined in Materials and Methods) and also for the FOG types of the trapezius (for all littermate groups) were not significantly different from 2/3. This suggested that the TSAs of these fibre types increased with the 2/3 power of the animal's liveweight. All the other types (i.e. the FOG and FG types of the deep and superficial portions of the semitendinosus and the FG types of the trapezius) grew at a significantly ($P < 0·025$) greater rate than the SO types. The TSAs of these other myofibres had the highest postnatal growth rates and in fact increased in direct proportion to liveweight.

DISCUSSION

The initial histochemical differentiation of muscle fibre types with respect to glycogen phosphorylase and succinate dehydrogenase was found to extend over a larger age range and up to a greater age (19 and 64 days, respectively) than has previously been determined for various muscles in the pig. The initiation of glycogen phosphorylase staining has been observed during the first week (in semitendinosus, Beermann, Cassens & Hausman, 1978) and up to 10 days (longissimus dorsi and the diaphragm, Davies, 1972) postnatally, while Ashmore, Addis & Doerr (1972*a*; cutaneous muscle and triceps brachii) found succinate dehydrogenase-negative (non-oxidative) fibres at 14 days and Davies (1972) demonstrated these fibres at 12 days

after birth. However, in the present investigation animals with a range in birthweights were specifically selected so that at any given liveweight the littermates of low birthweight were considerably older than those of a heavier birthweight. The results indicated that myofibre type differentiation, with respect to succinate dehydrogenase and glycogen phosphorylase, was weight, rather than age, specific, being more related to physiological than chronological age.

The process of fibre type conversion from fast to slow contractility, which was responsible for the increasing proportion of slow fibres in the growing muscle, involved the transitional myofibre types shown in Table 1. The transitional fibre types of the present study parallel the intermediate types documented by Suzuki & Cassens in pig (1980) and in sheep (1983) muscle. The population of FO fibres was apparently derived from fibres that had not yet adapted a combination of contraction speed and metabolic properties characteristic of a 'mature' fibre type. These fibres appeared to be the precursors of the transitional forms. Since the percentages of mature FOG and FG fibres were not attained in pigs until they had reached a liveweight of about 8·5 kg, after which the percentages of transitional fibre types were negligible (Table 2; Fig. 2), it seems that a contribution of FG or FOG fibres to the transitional myofibre population did not occur in either the semitendinosus or trapezius muscles of the pigs studied here.

Prior to the complete loss of alkaline ATPase stability the myosin ATPase of the FO fibres appeared to attain increasing degrees of acid stability (presented by Subtypes IOa, IOb, IOc and IOd; Table 1), a phenomenon documented by Kugelberg (1976) in rat soleus muscle, as well as by Suzuki & Cassens (1980) in pig muscles. These subtypes were already present in neonatal pig muscle which exhibited ratios of acid-stable to alkaline-labile fibres of between 1·6 and 4·9, illustrating the commencement of fibre type conversion prenatally (Beermann *et al.* 1978) in the pig. After a certain degree of acid stability had been achieved (demonstrated by Types IOb and IOd; Table 1) the alkaline stability of myosin ATPase appeared to decrease (seen in Types IOa and IOc; Table 1) until the acid and alkaline stability was characteristic of that demonstrated by SO fibres. Thus transitional fibres exhibiting a continuum of both acid and alkaline ATPase stabilities intermediate to FO and SO fibres were displayed on conversion to the latter fibre type.

The rates of change, with increasing liveweight, in the TSAs of the fast myofibres, especially the FG fibres, were generally greater (particularly in the semitendinosus) than those of the slow (SO) fibres (Table 3). It is interesting to relate these comparative differences in postnatal fibre size changes to the results of work carried out on the effects of undernutrition on the size of various muscle fibre types. A study conducted on protein-deprived rats from 6 to 25 weeks after birth (Oldfors, Mair & Sourander, 1983) shows that Type 1 (SO) and Type 2A (FOG) myofibres of the extensor digitorum longus muscle fail to grow while Type 2B (FG) fibres actually atrophy under this treatment. This dietary manipulation demonstrates the vulnerability of myofibres with a fast contraction speed to nutritional inadequacy. If postnatal undernutrition were to have similar effects in the pig as it does in the rat it would appear that relative postnatal myofibre growth rates are an indication of the comparative vulnerability of the different myofibre types to growth inhibition. The lack of differences in the relative growth rates of myofibre types between littermates of all three birthweight categories was verification of the adequate postnatal nutrition of even the most physically disadvantaged runt pigs.

The observation made, during the course of this work, that low birthweight pigs

have significantly larger myofibre TSAs in their semitendinosus and trapezius muscles (the degree of significance depending on the myofibre type and muscle, see Table 3) than their heavier littermates when considered at the same weight has also been noted by other authors. Hegarty & Allen (1978) found that, of the psoas major, semitendinosus, biceps brachii and longissimus dorsi muscles, the former two muscles contained fibres of greater ($P < 0{\cdot}05$) TSAs in runt pigs (average birthweight 810 g) than in their large littermates (average birthweight 1570 g) at 106 kg; the myofibre areas of the latter two muscles were not significantly different between siblings at the same liveweight. Likewise Powell & Aberle (1981) found that the fibre TSAs of the semimembranosus of runts (birthweight under 1000 g) were either equal to, or greater than, those of their heavier birthweight (over 1500 g) littermates at equal slaughter-weight. However, these authors did not attribute the fibre size differences to the disparate ages of similar slaughter-weight siblings of low and high birthweight. Since low birthweight pigs appear to maintain a significantly lower weight ($P < 0{\cdot}001$) through to 128 days they will be chronologically older at any specific weight. This highlights the very interesting and fundamentally important phenomenon that there must be an age-factor, unrelated to the growth-force of weight, influencing the TSA growth of myofibres. The relative differences in myofibre TSAs seen between littermates of the same liveweight is of particular significance in another aspect of muscle development, namely that concerning the TSA of slow muscle within the semitendinosus muscle which is discussed below.

The precise way in which the postural function of the semitendinosus adapts to the increasing liveweight of the pig through changes in the TSA of slow muscle was revealed in the present study. The increase was achieved, up to a liveweight of approximately 8·5 kg, by an increase in the percentage of slow fibres within the deep portion of the muscle together with an increase in the TSA of the individual fibres. The superficial portion of the semitendinosus contributed very few slow fibres (Table 2) and therefore a minimal supportive role to the muscle. Muscles, or parts of muscles concerned with a propulsive function, do not appear to show changes in their complement of slow fibres. This was exemplified by the superficial portion of the semitendinosus in the present study and in that of Sivachelvan & Davies (1981), and the biopsies (and therefore, presumably, the superficial portion) of the semitendinosus muscle of cattle in the work of Holmes & Ashmore (1972). Beyond a liveweight of 8·5 kg the only significant factor contributing to the increase in the area of slow muscle was the increasing TSA of the slow fibres. The results presented for the semitendinosus support the general hypothesis proposed by Davies (1972) that the TSA of a muscle varies, during growth, with the 2/3 power of liveweight while within the muscle the area occupied by slow fibres increases at a greater rate than the total TSA of the muscle. However, the differing regression coefficients determined for the longissimus dorsi (1·0; Davies, 1972) and for the semitendinosus in this paper (5/4) suggests that the slow muscle TSA increase, relative to liveweight, is a reflection of a specific muscle's unique adaptation to its comparative postural role within the body. This theory is supported by the relative values for the average alkaline ATPase-negative (slow) fibre type percentages for the two muscles; the longissimus dorsi of the mature Large White possesses less than half the number of slow fibres (18 %; Davies, 1972) possessed by the deep portion of the semitendinosus (38·5 %; Table 2). Consequently the supportive role of the former muscle is probably less than that of the latter.

The mechanism by which the muscle and its innervating neurons, which are

responsible for the muscle fibres' contractile properties (Close, 1965; Salmons & Sréter, 1976), acknowledge the need for an adaptation in the muscle's slow fibre compliment has yet to be elucidated. However, a notion of the way in which the supportive role of a muscle is maintained in accordance with the liveweight of an animal is given by the present findings. Littermates, when considered on a constant liveweight basis, had a similar number of slow fibres per metabolic bundle while possessing different slow fibre percentages. It therefore appears that it is possibly the number of slow fibres within a metabolic bundle, rather than the resultant percentage, that is regulated to maintain a constant relationship with liveweight. The percentage of slow fibres is a consequence of the number of fibres per metabolic bundle and the surrounding complement of fast fibres. The secondary/primary fibre number ratios, which are an indication of the total number of fibres per metabolic bundle, were significantly lower ($P < 0{\cdot}01$) in the low birthweight, runt littermates (Handel & Stickland, 1984) than in their heaviest birthweight littermates; the secondary/primary fibre number ratio, although lower in the small compared to large littermates, was not significantly different. Nevertheless, after acquisition of the number of slow fibres per metabolic bundle appropriate for the liveweight of the animal, both the small and runt littermates appeared to possess a lower complement of fast fibres and therefore a greater percentage of slow fibres (this produced a significant effect in the semitendinosus muscle). This, together with the greater mean TSA of the slow fibres within the deep portion of the semitendinosus of both small and runt littermates, contributed to the greater slow muscle TSA of these lower birthweight littermates relative to their large littermates. The resultant effect is indicative not only of a 'slower' semitendinosus muscle in the lower birthweight small and runt pigs but also higher oxidative and lower glycolytic capacities of this muscle (slow fibres were exclusively oxidative, Table 1).

The observations made as a result of this work indicate that relative weight at birth has a significant contributory effect on the subsequent comparative myofibre type composition of the muscle between pig littermates. It has also been revealed that age, in addition to liveweight, has an important influence on muscle growth and, likewise, its subsequent comparative myofibre composition. One of the cumulative effects of these phenomena is to produce significantly greater amounts of slow muscle in the smaller birthweight animals than in their larger siblings.

SUMMARY

Muscle growth and development was studied in 49 Large White pigs from a total of 17 litters. Representative large (mean birthweight of 1544 g), small (1144 g) and runt (776 g) littermates were selected and slaughtered at the same age, ages ranging from birth to 128 days. Fresh frozen, serial transverse sections taken from the semitendinosus and trapezius muscles of these animals were stained for the histochemical demonstration of acid and alkaline pre-incubated adenosine triphosphatase, succinate dehydrogenase and glycogen phosphorylase. Profiles of the muscle fibre types were compiled for each animal.

In both muscles the number of slow oxidative (SO) fibres, that were arranged together in groups within 'metabolic bundles', increased with growth. The transverse sectional area (TSA) of the semitendinosus muscle increased with the 2/3 power of liveweight whereas the area occupied by SO fibres increased at a rate significantly greater than 1·0 ($P < 0{\cdot}01$). Regression analysis revealed that the area of this muscle

occupied by SO fibres was greater ($P < 0{\cdot}001$) in runt and small littermates relative to their large littermates when they were compared at an equal liveweight. This greater TSA of the semitendinosus classified as 'SO' in lower birthweight pigs was the result of a combination of higher percentages ($P < 0{\cdot}05$) of SO fibres and significantly greater ($P < 0{\cdot}001$) SO fibre mean TSAs. The mean TSAs of all myofibre types were similar between littermates of the same age but most types were of greater TSA in the lower birthweight littermates when compared (by regression analysis) at the same liveweight suggesting that fibre TSA was age- rather than weight-related.

The higher percentage of SO fibres in the low birthweight pigs, when compared at an equivalent liveweight to their large littermates, appeared to be related to their affected secondary/primary fibre number ratio. This phenomenon, plus the data on the number of slow fibres per metabolic bundle, indicated that it was apparently the number of slow fibres per metabolic bundle which was regulated with liveweight gain rather than the resultant percentage of slow fibres within the muscle.

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REFERENCES

ASHMORE, C. R., ADDIS, P. B. & DOERR, L. (1972*a*). Postnatal development of muscle fibre types in domestic animals. *Journal of Animal Science* **34**, 37–41.

ASHMORE, C. R., ROBINSON, D. W., RATTRAY, P. & DOERR, L. (1972*b*). Biphasic development of muscle fibres in the fetal lamb. *Experimental Neurology* **37**, 242–255.

ASHMORE, C. R., ADDIS, P. B. & DOERR, L. (1973). Development of muscle fibres in the fetal pig. *Journal of Animal Science* **36**, 1088–1093.

BEERMANN, D. H., CASSENS, R. G. & HAUSMAN, G. J. (1978). A second look at fibre type differentiation in porcine skeletal muscle. *Journal of Animal Science* **46**, 125–132.

BURKE, R. E., LEVIN, D. N., ZAJAC, F. E., TASIRIS, P. & ENGEL, W. K. (1971). Mammalian motor units: physiological–histochemical correlation in three types in cat gastrocnemius. *Science* **174**, 709–712.

CLOSE, R. (1965). Effects of cross-union of motor nerves to fast and slow skeletal muscles. *Nature* **206**, 831–832.

DAVIES, A. S. (1972). Postnatal changes in the histochemical fibre types of porcine skeletal muscle. *Journal of Anatomy* **113**, 213–240.

EDSTRÖM, L. & KUGELBERG, E. (1968). Histochemical composition, distribution of fibres and fatigability of single motor units (anterior tibial muscle) of rat. *Journal of Neurology, Neurosurgery and Psychiatry* **31**, 424–433.

GUTH, L. & SAMAHA, F. J. (1970). Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28**, 365–367.

HANDEL, S. E. & STICKLAND, N. C. (1984). Muscle cellularity and its relationship with birth weight and growth. *Journal of Anatomy* **139**, 726.

HEGARTY, P. V. J. & ALLEN, C. E. (1978). Effect of pre-natal runting on the post-natal development of skeletal muscles in swine and rats. *Journal of Animal Science* **46**, 1634–1640.

HOLMES, J. H. G. & ASHMORE, C. R. (1972). A histochemical study of development of muscle fibres in normal and double-muscled cattle. *Growth* **36**, 351–372.

KUGELBERG, E. (1976). Adaptive transformation of rat soleus motor units during growth. Histochemistry and contraction speed. *Journal of Neurological Science* **27**, 269–289.

NACHLAS, M. M., TSOU, K. C., DESOUZA, E., CHENG, C. S. & SELIGMAN, A. M. (1957). Cytochemical demonstation of succinic dehydrogenase by the use of a new *p*-nitrophenyl substituted ditetrazole. *Journal of Histochemistry and Cytochemistry* **5**, 420–436.

OLDFORS, A., MAIR, G. P. & SOURANDER, P. (1983). Muscle changes in protein deprived young rats. A morphometrical, histochemical and ultrastructural study. *Journal of Neurological Science* **59**, 291–302.

PETER, J. B., BARNARD, R., EDGERTON, V. R., GILLESPIE, C. A. & STEMPEL, K. E. (1972). Metabolic profiles of three fibre types of skeletal muscle in guinea pigs and rabbits. *Biochemistry* **11**, 2627–2633.

POWELL, S. E. & ABERLE, E. D. (1981). Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. *Journal of Animal Science* **52**, 748–756.

QUENOUILLE, M. H. (1969). *Introductory Statistics*, 2nd ed. London: Pergamon Press.

SALMONS, S. & SRÉTER, F. A. (1976). Significance of impulse activity in the transformation of skeletal type. *Nature* **263**, 30–34.

SIVACHELVAN, M. N. & DAVIES, A. S. (1981). Antenatal anticipation of postnatal muscle function. *Journal of Anatomy* **132**, 545–555.

SRÉTER, F. A., SEIDEL, J. C. & GERGELY, J. (1966). Studies on myosin from red and white skeletal muscles of the rabbit. I. Adenosine triphosphatase activity. *Journal of Biological Chemistry* **241**, 5772–5776.

STAUN, H. (1963). Various factors affecting number and size of muscle fibres in the pig. *Acta Agriculturae Scandinavica* **13**, 293–322.

STICKLAND, N. C. & GOLDSPINK, G. (1973). A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16**, 135–146.

SUZUKI, A. & CASSENS, R. G. (1980). A histochemical study of myofibre types in muscle of the growing pig. *Journal of Animal Science* **51**, 1449–1461.

SUZUKI, A. & CASSENS, R. G. (1983). A histochemical study of myofibre types in serratus ventralis thoracis muscle of sheep during growth. *Journal of Animal Science* **56**, 1447–1458.

TAKEUCHI, T. (1956). Histochemical demonstration of phosphorylase. *Journal of Histochemistry and Cytochemistry* **4**, 84.

TAKEUCHI, T. & KURIAKI, H. (1955). Histochemical detection of phosphorylase in animal tissues. *Journal of Histochemistry and Cytochemistry* **3**, 153–160.

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The effects of low birthweight on the ultrastructural development of two myofibre types in the pig

S. E. HANDEL* AND N. C. STICKLAND†

Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh, Scotland and †Department of Anatomy, The Royal Veterinary College, Royal College Street, London, NW1 0TU

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INTRODUCTION

The ultrastructural characteristics of muscle fibre types of adult muscle are well defined in many mammals including humans (Sjöström, Kidman, Henriksson Larsén & Ängquist, 1982), guinea-pigs (Eisenberg, Kuda & Peter, 1974; Eisenberg & Kuda, 1976) and rats (Gottschall, 1980). The criteria for myofibre typing by ultrastructural characteristics, from longitudinal sections of muscle in particular, are well documented and include *Z*-disc thickness (Gauthier, 1968; Salmons, Gale & Sréter, 1978), amount and distribution of sarcoplasmic reticulum (Padykula & Gauthier, 1970; Ninomiya, Echeverría & Vázquez-Nin, 1981), quantity and internal structure of mitochondria (Gauthier, 1968) and also myofibrillar array (Salmons *et al.* 1978; Ninomiya *et al.* 1981). Changes in the subcellular components of mature muscle fibres may be experimentally induced by both electrical (Eisenberg & Salmons, 1981) and mechanical (Ashmore & Summers, 1981) stimulation. Some of these studies have helped to elucidate the mechanisms involved in the functional adaptation of muscle. However, there appear to be no comprehensive, comparative studies of the development of different muscle fibre types, from birth to maturity, which quantify the changes in subcellular components.

Comprehensive quantitative studies provide an invaluable reference in investigations of muscle pathology, aiding the early identification of myopathies, and enabling the evaluation of the rate and extent of their manifestation in the affected tissue. The electron microscope was employed in the present study of porcine muscle development in order to perform a stereological analysis of the ultrastructural changes occurring during growth in the two most diverse muscle fibre types (slow oxidative and fast glycolytic). This method was used to investigate the myofibrillar, mitochondrial and lipid droplet percentage volumes in the two myofibre types. These subcellular organelles were chosen in order to ascertain the general morphological changes occurring in different myofibres with growth after birth. In addition, the data obtained would reveal whether low birthweight within pig litters had any permanent effect on the myofibrillar protein content (a measure of muscle strength: Helander & Thulin, 1962; Goldspink, 1965) or the amount of mitochondria and lipid droplets present (which together would probably give a relative assessment of the capacity for oxidative metabolism) in the muscles of the affected animals.

* Present address: Muscle Biology Laboratory, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

MATERIALS AND METHODS

A total of 32 pigs, chosen from 11 pure bred Large White litters, was used to investigate the effects of birthweight on the subsequent postnatal development of two diverse muscle fibre types. These pigs were selected on the basis of their weight at birth, with a maximum of three representative animals being chosen from each litter including the largest male, smallest normal (not less than 1000 g) male and the runt (950 g or less). 'Runt' littermates were defined as those born of a weight below that considered 'critical' (about 1000 g), with respect to consequential development, by Hegarty & Allen (1978), and Powell & Aberle (1980), while 'small normal' littermates were those born slightly heavier than this critical weight. Pigs were weaned at between 4 and 5 weeks of age. Representative littermates from selected litters were weighed and slaughtered by electrical stunning followed by exsanguination at one of the following ages: 0, 1, 2, 4, 6, 9, 15, 27, 33, 46 and 84 days. The left semitendinosus muscle was dissected from the carcass and samples were excised from the midbelly for transmission electron microscopy. Longitudinal strips (approximately 1 mm × 3 mm) of muscle fibres were dissected from both the deep and superficial portions of the muscle, and fixed within 30 minutes of slaughter in 5 % glutaraldehyde in 0·1 M sodium cacodylate buffered at pH 7·2 for 2 hours. This was followed by two washes in buffer, post-fixation in 1 % osmium tetroxide in buffer for 1 hour, two washes in double distilled water and, finally, dehydration through graded acetone. The samples were embedded in Araldite. Transverse sections were cut to a thickness of 60–80 nm (see later) with a Reichert OMU3 microtome and stained with uranyl acetate (20 minutes) and lead citrate (4–5 minutes). These ultrathin sections were examined with a Philips 400 electron microscope at an accelerating voltage of 100 kV.

Selection of samples

The two myofibre type populations (oxidative and non-oxidative), chosen for stereological analysis of their ultrastructural composition, were selected from muscle samples by applying knowledge concerning the morphology of the muscle, with respect to fibre type location, gained from personal observations of the histochemical demonstration of myofibre types within the muscle; full details of the histochemical investigations of the semitendinosus muscle will be given in another communication (Handel & Stickland, in preparation). Non-oxidative fibres (or, in the case of immature muscle where all myofibres exhibit oxidative staining, the progenitors of non-oxidative fibres) were invariably located at the periphery of fascicles in the superficial portion of the muscle. Oxidative fibres were consistently demonstrated, from birth to maturity, at the centre of fascicles in the deep portion of the muscle. The choice of oxidative and non-oxidative fibres from these locations meant that the non-oxidative fibres were from a population of histochemically determined fast fibres while the oxidative fibres were invariably defined as slow-contracting. Despite these further differences between the two chosen fibre populations their respective oxidative capacities produced the most prevalent ultrastructural disparities between them. Therefore, although the fibre type groups investigated are termed oxidative and non-oxidative it must be stressed that they are representative of the slow oxidative and fast glycolytic fibre populations, respectively, of the semitendinosus muscle.

Stereological methods

Stereology relates two dimensional profiles of cell components seen in sections to their three dimensional structures in the original tissue. By the criteria of geometric probability the chance of finding organelle profiles in a section will equal that of finding the total volume of the organelle in the cell volume, a principle which permits the employment of point-counting techniques (Weibel, Kistler & Scherle, 1966). Specifically the volume fraction of an organelle (V_0) in a myofibre is estimated by the ratio of the number of test points falling on the organelle (P_o) to those falling on the myofibre (P_m), giving $V_0 = P_o/P_m$. Muscle tissue, which is composed of fully orientated muscle fibres, is anisotropic (Elias & Hyde, 1980) which means that it displays a periodicity in the arrangement of some of its cytological structures. The myofibrils within a muscle fibre consist of recurring units (sarcomeres) and a concentration of large mitochondria is associated with the I band of each sarcomere while small mitochondria are concentrated at the A band with the largest number found in an annulus within 1 μm of the sarcolemma (Eisenberg *et al.* 1974). The sarcoplasmic reticulum is probably the organelle whose appearance is most affected by the plane of sectioning. Weibel (1972) has suggested that oblique muscle sections, at a minimum of 70° to the long axis, should be taken for adequate stereological analysis of this organelle. However, the sarcoplasmic reticulum is not being considered here and, in any event, it was found that any transverse section contained some myofibres cut obliquely and that within each myofibre the myofibrils were not perfectly aligned so were sectioned at different levels of the sarcomere (personal observations). Therefore it was considered that the sectioning standards required of this anisotropic tissue were met quite satisfactorily by 'transverse' sectioning. The chosen thickness of the sections (60–80 nm) was less than mitochondrial diameter (Weibel *et al.* 1966), a precaution which prevents an over-estimation of the volume fraction of mitochondria.

Electron micrographs were taken of individual muscle fibres at a magnification of $\times 6000$ and were printed to give a final magnification of $\times 12000$. When myofibres failed to be completely represented by one electron micrograph, micrographs were taken across, and through the centre of, the fibre to create a montage on which a sector could be drawn. A sector is representative of the whole transverse sectional area of the fibre and allows for the localisation of mitochondria within circular zones (Eisenberg *et al.* 1974). In neonatal pig muscle the myofibrils were not evenly dispersed throughout the myofibres and so electron micrographs were taken to allow stereological analysis of the whole transverse sectional area of the myofibre rather than of a sector which would have been unrepresentative in such instances.

The number of myofibres of each type sampled per animal was determined so as to produce volume estimates of a value within the limits of $\pm 10\%$ (Eisenberg *et al.* 1974) of the population mean for a sample of 15 myofibres. By this method the minimum sample size was evaluated at 10 myofibres. A 1 cm square test grid drawn on an acetate sheet (Eisenberg & Salmons, 1981) was used for point-counting and hence the estimation of the volume density (expressed as a percentage) of myofibrils, mitochondria and lipid droplets for both the oxidative and non-oxidative fibre populations of the semitendinosus from pig littermates.

Regression analysis of myofibre organelle percentage volumes against liveweight and/or age was conducted to quantify postnatal changes. Paired observations were analysed (in the form of a paired t-test) to test the significance of the difference

between the mean percentage volumes of organelles of the two myofibre types (by the pairing of organelle volume fractions of oxidative and non-oxidative fibres from the same animal for all littermates together, regardless of their birthweight) and, also, to establish variations in these parameters between groups of littermates of different birthweights (by pairing animals of different birthweights from the same litter).

RESULTS

The mean birthweights (±S.D.) of the large, small, and runt littermates were 1559 g ± 225 ($n = 11$), 1147 g ± 217 ($n = 9$), and 758 g ± 120 ($n = 12$), respectively, the average weight difference between each group being statistically significant ($P < 0.01$). Of the 11 selected litters two failed to present pigs that could be defined as 'small' littermates and one of these litters contained two runts which accounts for the uneven number of animals in each birthweight category.

The data obtained from the stereological analysis of both oxidative and non-oxidative fibres from all 32 pigs investigated are represented in Figures 2 and 3. At each given age (Fig. 2) there are usually 3 points for each fibre type; each point represents one littermate. The following analysis of results is based on the data given in both Figures 2 and 3.

Comparison of the ultrastructure of two different myofibre types

From birth to 4 days of age the mean myofibrillar percentage volume of non-oxidative fibres (21·2 %) was significantly ($P < 0.001$) less than that of oxidative fibres (46·0 %) (Fig. 1 *a*, *b*). From 4 to 6 days of age (corresponding to liveweights of about 1350 to 2200 g) the increasing percentage myofibrillar volume of the non-oxidative fibres exceeded that of the oxidative fibres in all littermates except in two severely runted pigs which were noted for their lack of liveweight gain to slaughter at 9 and 15 days of age. These pigs had extremely low relative birthweights, 50 % less than their large littermates and more than 2·5 S.D. below the mean litter-weight. The mean myofibrillar percentage volume of non-oxidative fibres from pigs older than 6 days (and of a liveweight exceeding 2200 g) was 62·9 %, significantly ($P < 0.001$) greater than that of the oxidative fibres (51·9 %) (Fig. 1 *c*, *d*); these two fibre types therefore showed a reversal of their relative myofibrillar percentage volumes when the pigs exceeded 4 days of age. The change in the percentage volume of myofibrils between 46 and 84 days was only slight which suggested that the mature percentage values of myofibrillar volumes for the two myofibre types had been attained by 46 days to give mature values of approximately 56·3 % and 66·9 % for oxidative and non-oxidative fibres, respectively.

The increase in the percentage volume of myofibrils was significantly correlated with age (Fig. 2) and with liveweight (Fig. 3) in both the oxidative and non-oxidative fibres. The correlations of the percentage volume of myofibrils in the oxidative

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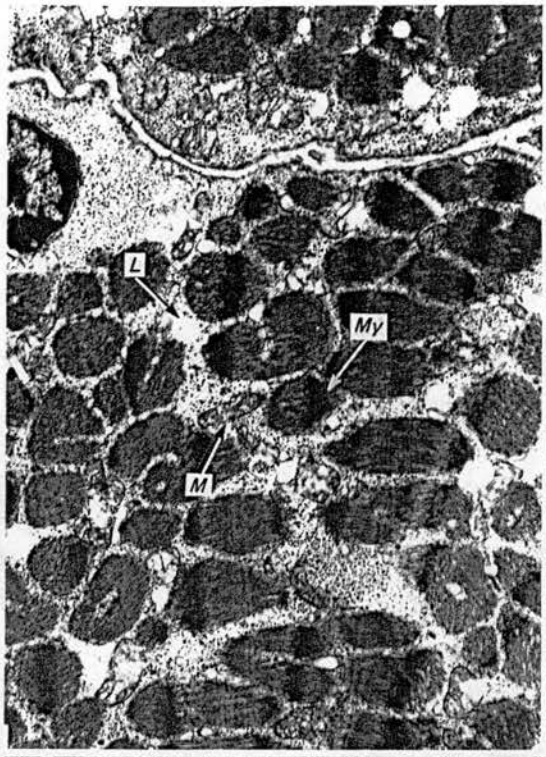
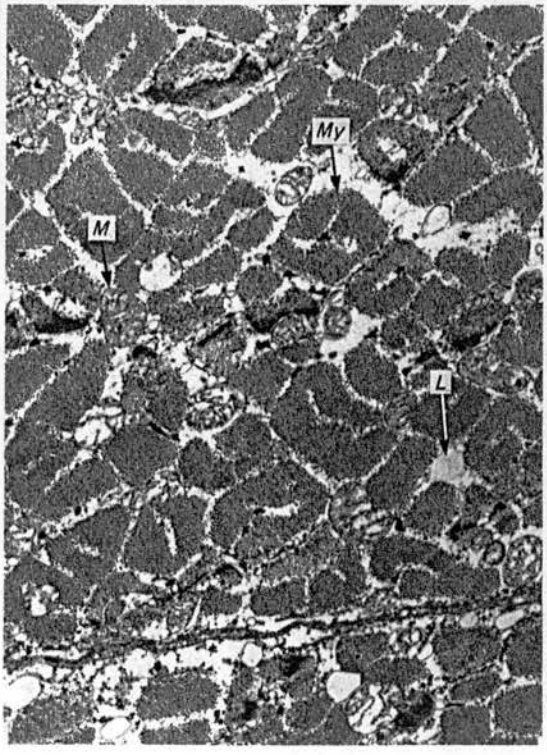
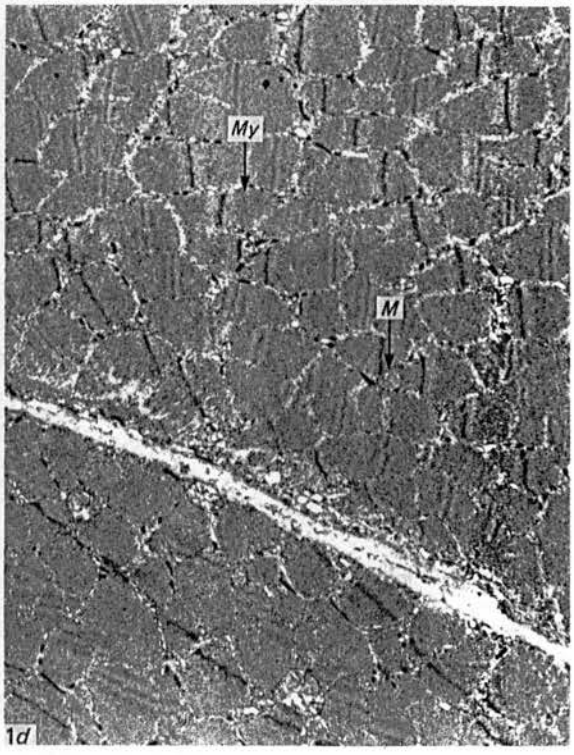
Fig. 1 (*a–d*). Oxidative and non-oxidative myofibre type ultrastructure at birth and 84 days. *My*, myofibrils; *M*, mitochondria; *L*, lipid droplets. (*a*) Oxidative fibre from 'deep' portion of m. semitendinosus of runt littermate at birth (liveweight of 0·9 kg). (*b*) Non-oxidative fibre from 'superficial' portion of m. semitendinosus of runt littermate at birth (liveweight of 0·9 kg). (*c*) Oxidative fibre from 'deep' portion of m. semitendinosus of runt littermate at 84 days (liveweight of 28·3 kg). (*d*) Non-oxidative fibre from 'superficial' portion of m. semitendinosus of runt littermate at 84 days (liveweight of 28·3 kg). × 7500.



1b





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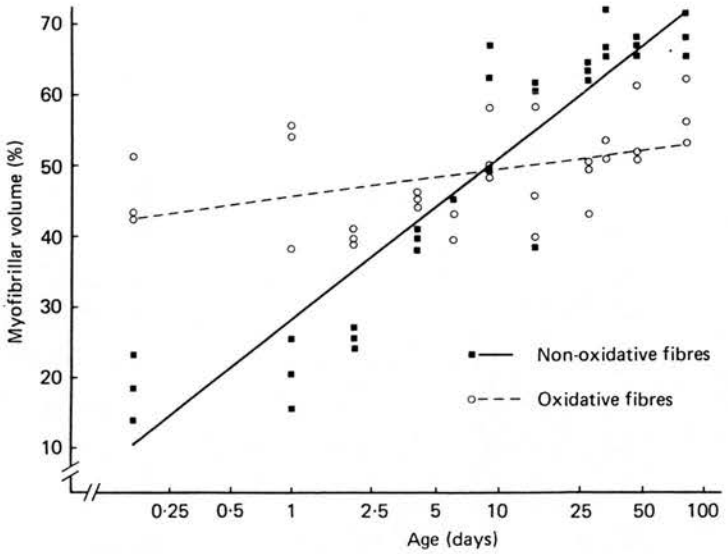

Fig. 2. Myofibrillar percentage volume of oxidative and non-oxidative fibres (from 'deep' and 'superficial' portions, respectively) of the semitendinosus muscle of individual littermates plotted against the natural logarithm of their age. Regression equations: oxidative fibres, $Y = 1{\cdot}6 \ln X + 45{\cdot}7$; non-oxidative fibres, $Y = 9{\cdot}9 \ln X + 28{\cdot}0$ (Y = myofibrillar % volume, X = age in days).

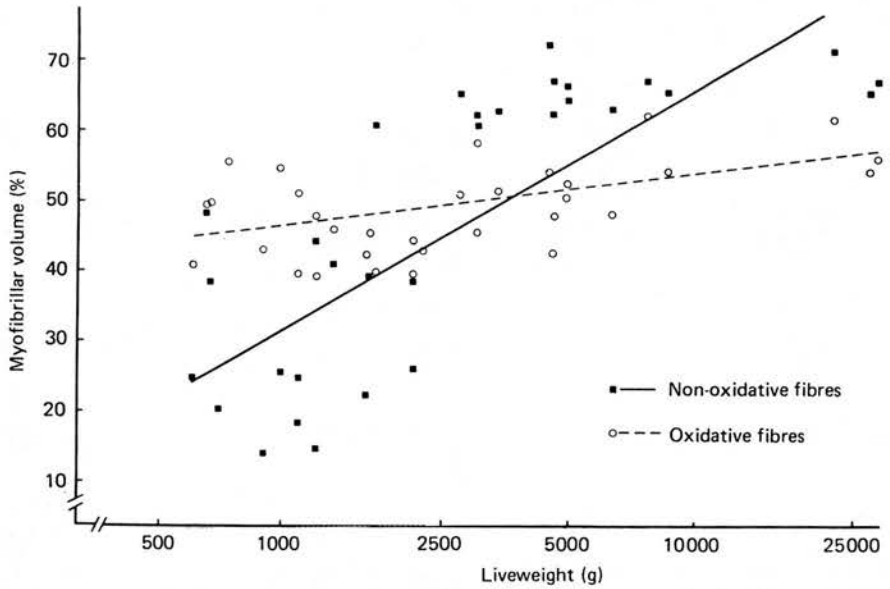

Fig. 3. Myofibrillar percentage volume of oxidative and non-oxidative fibres (from 'deep' and 'superficial' portions, respectively) of the semitendinosus muscle of individual littermates plotted against the natural logarithm of their liveweight. Regression equations: oxidative fibres, $Y = 2{\cdot}8 \ln X + 27{\cdot}1$; non-oxidative fibres, $Y = 14{\cdot}6 \ln X - 68{\cdot}7$ (Y = myofibrillar % volume, X = liveweight in grams).

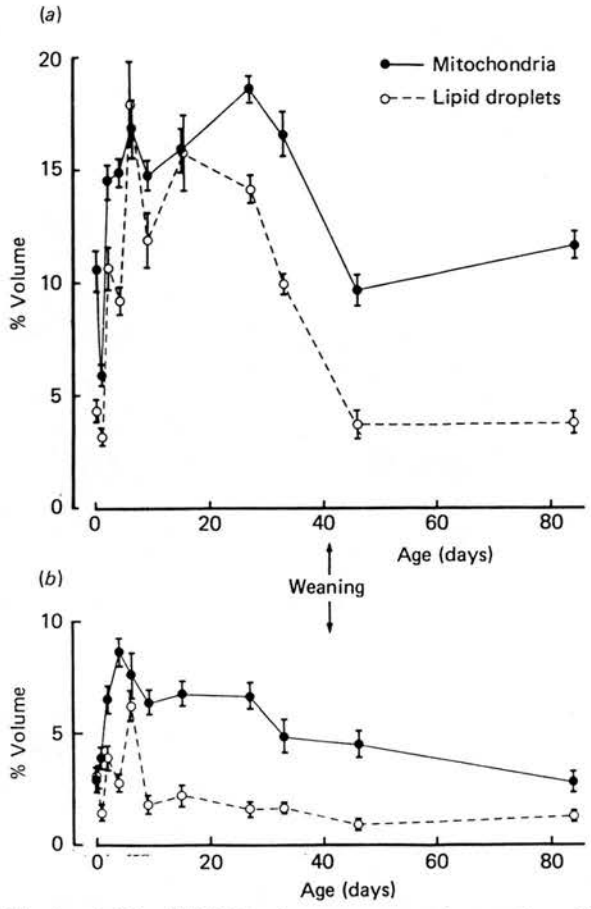

Fig. 4 (*a–b*). Mitochondrial and lipid droplet percentage volumes of myofibres, expressed as an average value for fibres from large, small and runt littermates against age. (*a*) Oxidative fibres, from 'deep' portion of semitendinosus. (*b*) Non-oxidative fibres, from 'superficial' portion of semitendinosus.

fibres with ln (age) ($r = 0{\cdot}445$, $P < 0{\cdot}01$) and ln (liveweight) ($r = 0{\cdot}450$, $P < 0{\cdot}02$) were not significantly different. The change in the percentage volume of myofibrils in the non-oxidative fibres showed a significantly ($P < 0{\cdot}025$) greater correlation with ln (age) than with ln (liveweight), unlike the oxidative fibres. In addition, the myofibrillar percentage volumes of the non-oxidative fibres were found to be more highly correlated with both ln (age) ($r = 0{\cdot}924$, $P < 0{\cdot}001$) and ln (liveweight) ($r = 0{\cdot}727$, $P < 0{\cdot}001$) than those of the oxidative fibres.

The mitochondria and lipid droplet percentage volumes of the oxidative and non-oxidative myofibres were significantly different ($P < 0{\cdot}001$) at all ages from birth to 84 days; the oxidative fibres possessed a greater percentage volume of both these subcellular components (illustrated by the electron micrographs in Figure 1 and the graphs in Figure 4). The percentage volume difference in mitochondria between the two fibre types was more or less consistent (at about 8 %) throughout, even during the rise in mitochondrial content between 2 and 33 days of age. During this period the mitochondrial percentage volume of oxidative and non-oxidative fibres increased from mean values of 8·3 % and 3·4 %, respectively, before 2 days, to 16·0 % and

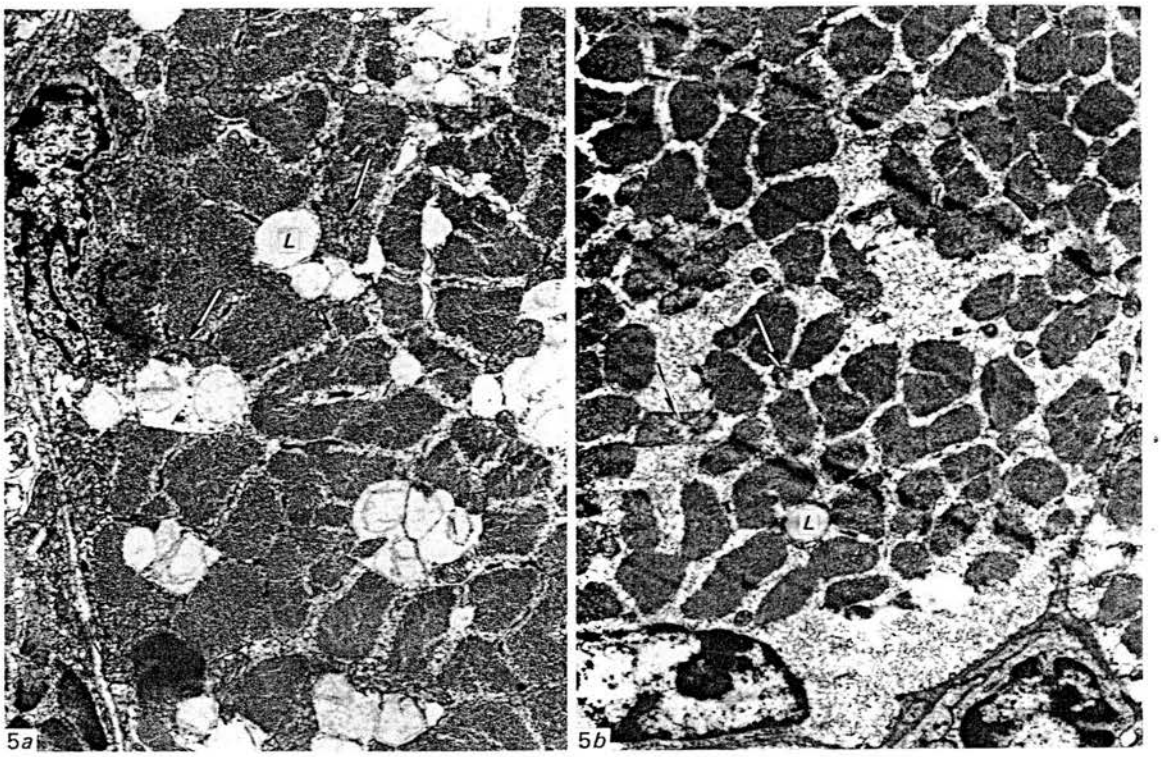

Fig. 5 (*a–b*). Oxidative myofibre ultrastructure from the 'deep' portion of semitendinosus of littermates before and after weaning: arrows indicate mitochondria; *L*, lipid droplets. (*a*) Before weaning. Large littermate, 33 days old. Mean % volumes; myofibrillar, 51·3 %; mitochondrial, 15·0 %; lipid droplets, 10·5 %. (*b*) After weaning. Large littermate, 46 days old. Mean % volumes; myofibrillar, 52·8 %; mitochondrial, 7·6 %; lipid droplets, 3·7 %. × 7000.

6·7 %, respectively, at 33 days. In the oxidative myofibres, changes in the mean percentage volume of mitochondria were mirrored by changes in the mean percentage volume of lipid droplets, but this phenomenon was less markedly exhibited by the non-oxidative fibres. Consequently, the lipid droplet percentage volume difference between the oxidative and non-oxidative fibre types was greatest between 2 and 33 days. As demonstrated by Figure 4*a*, during this age-range the percentage lipid droplet volume of oxidative fibres more than trebled from a mean value of 3·8 % (at 0 and 1 days) to a mean value of 13·1 % (between 15 and 27 days). However, in the non-oxidative fibres (Fig. 4*b*) there was little change between 2 and 33 days (the lipid droplet percentage volume at 6 days in Figure 4*b* which suggests an apparent rise was from one littermate only, and was therefore probably not truly representative of non-oxidative fibres from pigs of this age; other values in this

Fig. 6 (*a–c*). Myofibrillar hypoplasia of the myofibrils from the 'superficial' portion of the semitendinosus muscle of a severely runted pig at 15 days postnatally. *My*, myofibrils; *M*, mitochondria; *L*, lipid droplets. (*a*) Low power electron micrograph to demonstrate the consistent occurrence of myofibrillar hypoplasia. × 3000. (*b*) Higher magnification of a non-oxidative fibre of the severely runted pig (liveweight of 675 g). Mean % volumes; myofibrillar, 38·8 %; mitochondrial, 6·6 %; lipid droplet, 1·4 %. (*c*) Non oxidative myofibre of the runt's small littermate (liveweight of 2750 g) to illustrate myofibrillar content of a 'normal' fibre. Mean % volumes; myofibrillar, 60·8 %; mitochondrial, 6·7 %; lipid droplet, 3·5 %. × 7000.

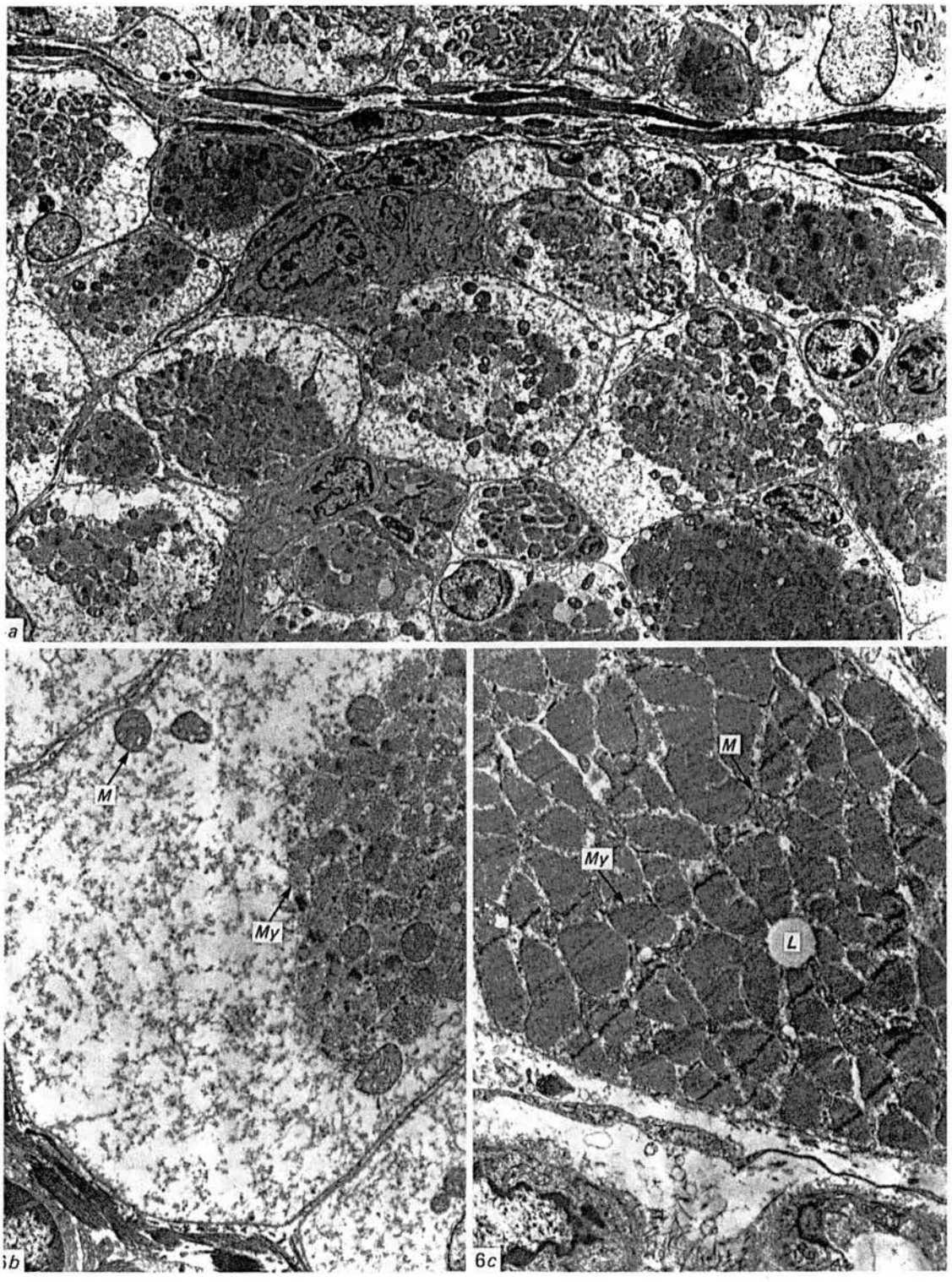
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Figure were means of littermates). After weaning, myofibres possessed much lower percentage volumes of mitochondria and lipid droplets (Fig. 5). Between the littermates of 46 days (weaned at 41 days of age) and 84 days of age there appeared to be little difference in the percentage volumes of mitochondria and lipid droplets, which gave mean values of 10·6 % and 3·6 % for oxidative fibres, and 3·6 % and 1·1 % for non-oxidative fibres, respectively.

Comparison of myofibre type ultrastructure between littermates

Statistical analysis of the data showed that there was no significant difference in the myofibre percentage volumes of either myofibrils, mitochondria or lipid droplets between large and small, small and runt or large and runt littermate category comparisons, with one exception; overall, the percentage myofibrillar volume of the oxidative fibres from the small littermates was higher ($P < 0{\cdot}025$) than in the large littermates, by an average of 9·5 %.

On consideration of individual litters there were specific instances of high variation in the myofibrillar percentage volumes of non-oxidative fibres between some littermates. The two, previously mentioned, severely runted littermates presented myofibrillar hypoplasia (an apparent lack of myofibrils) in their non-oxidative fibres (Fig. 6). These runt littermates possessed myofibrillar percentage volumes with an average value of approximately 68 % of the value for their large littermates. Also these runts had myofibrillar percentage volumes in the non-oxidative fibres of about 76 % of the value for their oxidative fibres (this value for their heavier birthweight littermates, classified under both the small and large birthweight categories, was approximately 128 %). The relative non-oxidative/oxidative myofibrillar percentage volumes of these extremely runted pigs was reminiscent of that seen in pigs of less than 6 days of age. The mitochondrial and lipid droplet percentage volumes of these severely runted animals did not, however, appear to be abnormally different from those of their heavier birthweight littermates.

DISCUSSION

In the present study the myofibrillar percentage volume was found to be different between muscle fibres from neonatal and relatively mature pigs. In addition the two myofibre type populations studied exhibited different rates of myofibrillar proliferation, a phenomenon that apparently proceeds by way of splitting along the length of the myofibrils with the eventual separation of the two fractions (Goldspink, 1970). The rate of myofibrillar proliferation in the non-oxidative fibres far exceeded that in the oxidative fibres, a fact that accentuates the rather more dynamic nature of the postnatal growth of non-oxidative fibres in comparison with oxidative fibres (Handel and Stickland, submitted for publication). Coupled with this was the evident lack of myofibrils in the non-oxidative fibres relative to the oxidative fibres of pigs prior to 6 days of age. It therefore appeared that the development of oxidative fibres, with respect to myofibrillar protein content, preceded that of the non-oxidative fibres before birth.

It is a well accepted concept that undernutrition stunts or reduces the growth of tissues in the reverse order of their development, i.e. as bone is the first tissue to reach its maximum rate of growth, followed by muscle and lastly fat (McMeekan, 1940), bone will be the least affected tissue, muscle more affected and fat most

affected if the animal suffers a period of undernutrition *in utero*. Extending this theory to parts of a particular tissue, in this case muscle, it seems feasible to hypothesise that the oxidative fibres within a muscle, which develop ultrastructurally prior to the non-oxidative fibres, will be less affected by growth retardation imposed by inadequate nutrition. This was, in fact, found by Oldfors, Mair & Sourander (1983) to be the case in the extensor digitorum longus muscle of young rats after protein deprivation. While the oxidative (slow oxidative and fast oxidative glycolytic) types failed to grow, the non-oxidative (fast glycolytic) fibres actually atrophied under this treatment. The results of the present study, therefore, appear to suggest that the comparative rate of myofibrillar development between oxidative and non-oxidative fibres might, in fact, be the basis for a differential response of the two fibre types to the effects of undernutrition in the pig. Unfortunately experiments carried out to ascertain the effects of postnatal undernutrition on the relative sizes of different fibre types appear to be limited to rats (Bedi *et al.* 1982; Oldfors *et al.* 1983) with no results available for such manipulations in pigs to compare with the ultrastructural observations made here.

The existence of a comparatively low percentage of myofibrils in the myofibres of neonatal pigs, younger than 6 days, substantiates the results of Bradley, Ward & Bailey (1980). These workers observed the ultrastructure of fibres from the adductor muscle and from the sartorius of normal and Splayleg pigs; the adductor was chosen by virtue of its reduced function in Splayleg pigs which prevents the hindlimbs of the affected pig from maintaining a normal stance (Thurley, Gilbert & Done, 1967; Deutsch & Done, 1971; Ward & Bradley, 1980). Bradley *et al.* (1980) discovered that the myofibrillar hypoplasia of both the adductor and the sartorius was not confined to pigs exhibiting Splayleg symptoms, as was once thought (Thurley *et al.* 1967; Deutsch & Done, 1971), but was, in fact, a normal occurrence in pigs after birth until one week of age. Although a quantitative assessment of the degree of myofibrillar hypoplasia was not established there appear (from the electron micrographs presented in the article of Bradley *et al.* 1980) to be no profound differences between the myofibrillar content of the muscle fibres from normal and Splayleg pigs. This example does, however, illustrate the need for comprehensive quantitative studies to assist our understanding of diseases of the musculature.

The existence of a significantly greater correlation between the non-oxidative myofibrillar percentage volume and age ($r = 0{\cdot}924$) than liveweight ($r = 0{\cdot}727$) suggests that there was an age-related factor in the attainment of myofibrillar percentage volume in the non-oxidative fibres distinct from that associated with the liveweight increase that occurs with age. It is therefore apparent that the attainment of myofibrillar percentage volume is not primarily weight-related. It has been found (by Helander & Thulin, 1962) that a direct relationship exists between the maximal isometric tension in the skeletal muscles of cat and rabbit limbs and their total myofilament transverse sectional area (determined biochemically). This relationship was further substantiated by the decrease in maximum tetanus tension produced in the muscles of mice after partial starvation for 7 days; the decrease in strength was proportional to the decrease in size and number of the myofibrils (Goldspink, 1965). This suggests that the muscles of young (or exceptionally growth retarded) pigs are relatively weaker per unit muscle transverse sectional area than the muscles of older pigs (that are showing liveweight gain) due to their comparatively lower myofibrillar percentage volumes. Maximum muscle strength is realised in a muscle when myofibrillar percentage volume has reached an upper limit which then remains constant

with growth. This upper limit appeared to be reached in the semitendinosus muscle of pigs over 46 days of age.

The failure of the myofibres of the low birthweight littermates (that were exhibiting normal growth rates) to present significant deficiencies in their percentage myofibrillar volumes made it apparent, in view of the reputed relationship between myofibrillar transverse sectional area and muscle strength, that there was no evident effect on the strength per-unit-transverse sectional area of the muscle of light, relative to heavy birthweight pigs. However, when severe prenatal growth retardation was associated with a lack of postnatal liveweight gain the muscles of the affected pig presented a lower degree of myofibrillar proliferation than was characteristic for their age and, therefore, they presumably possessed muscles which were unable to exert equivalent forces to those of their large littermates.

Oxidative and non-oxidative fibres both exhibited a considerable rise in mitochondrial percentage volume within a few days after birth; the mitochondrial percentage volume at birth, and the increase thereafter, was highest in the oxidative fibres. This phenomenon is indicative of an increased mitochondrial size and/or an enhanced mitochondrial proliferation which proceeds by fission. Mitochondrial percentage volume was more or less sustained in proportion to fibre transverse sectional area (i.e. mitochondrial percentage volume remained approximately constant) between about 4 and 33 days postnatally in both fibre types (Fig. 4). Between 33 and 46 days mitochondrial percentage volume appeared to decline in relation to fibre transverse sectional area, a proportion that remained constant thereafter; this is indicated by the lack of a marked alteration in mitochondrial percentage volume after 46 days.

The mitochondrial percentage volume in both oxidative and non-oxidative fibres appeared to decline just before weaning which is suggestive of a change in cellular metabolism related to dietary adjustments; the high-fat milk diet consumed from birth was gradually replaced by the voluntary consumption of creep-feed prior to weaning, allowing a gradual adjustment to the solid diet after weaning.

In the oxidative fibres the mitochondrial percentage volume changes were paralleled by the percentage volume changes in lipid droplets. The close structural association between mitochondria and lipid droplets (Gauthier & Padykula, 1966) is due to the use, by enzymes localised within mitochondria, of lipid substrate for the oxidative reactions performed by these enzymes to provide energy (in the form of ATP) for cellular metabolic processes. A relationship between plasma lipid values and muscle oxidative capacity is suggested by the work of Kraeling, Rampacek, Campion & Richardson (1978) who found that blood triglyceride bore a direct relationship with muscle succinate dehydrogenase activity in pig fetuses. The percentage volume of lipid droplets in the oxidative fibres exhibited a considerable rise between birth and weaning which was probably a reflection of the high-fat milk diet, and therefore higher plasma lipid content, of the suckling pigs. Bradley *et al.* (1980) also found that intrafibre lipids increased after one day of age. Moody *et al.* (1978) demonstrated a dramatic rise in the chloroform-extracted muscle lipid concentration from 1 to 3 days and thence, until 28 days, it remained constant. Despite this Moody *et al.* (1978) found that the actual population of fibres staining positively for intrafibre lipid (demonstrated by oil red O) was reduced with increasing age while there was a gradual increase in the amount of interfascicular lipid. Suzuki (1974) suggested that the oxidative fibres (exhibiting a positive reaction for β-hydroxybutyrate dehydrogenase) of starved sheep possessed the ability to include fatty droplets, mobilised

from body stores, to compensate for the lack of dietary energy intake. The apparent reflection (in the present study and that of Suzuki (1974)) of high plasma fat content within the oxidative fibres alone might be due to the relatively higher capillary density and blood flow of oxidative (slow oxidative) fibres than non-oxidative (fast glycolytic) fibre types (Mackie & Terjung, 1983) thus transporting a larger amount of fat to these oxidative fibres, or to the capacity of this fibre type to utilise lipid, as suggested by Suzuki (1974).

The mitochondrial percentage volume results of the present study tend to suggest that the ultrastructural diversification of fibre types may not parallel their acquisition of histochemical differentiation with respect to oxidative enzymes. Oxidative fibres from the deep portion of the semitendinosus muscle maintained their positive succinic dehydrogenase staining at all ages investigated and possessed mitochondrial percentage volume estimates in excess of 5 % throughout. However, the non-oxidative fibres of the superficial portion of this muscle were shown, histochemically, to exhibit a capacity for oxidative metabolism at birth, eventually losing it between 6 and 19 days of age (Handel & Stickland, submitted for publication). These observations do not correspond with the mitochondrial percentage volume estimates determined for non-oxidative fibres which presented values at birth, when these fibres were SDHase-positive, similar to values at 84 days when succinate dehydrogenase staining was negligible. It would appear therefore that the mitochondrial content of a myofibre does not necessarily reflect absolute succinic dehydrogenase activity at birth. Krieger, Tate, McMillin-Wood & Booth (1980) showed that subsarcolemmal and intermyofibrillar mitochondria may have different succinic dehydrogenase activities. They also found that functional activity may alter the succinic dehydrogenase activity of both types of mitochondria although it appears that the enzyme activity is not a rate-limiting factor as far as respiratory activity is concerned. It is possible that the proportions and enzyme activities of the two different types of mitochondria may change with age. It is also possible that these changes may differ between the two functionally distinct muscle fibre types studied here. Clearly the apparent anomalies seen here within the non-oxidative fibres require further investigation using biochemical methods such as those employed by Krieger *et al.* (1980).

One of the most interesting points to come out of this work was the discovery that low birthweight pigs, with the exception of those of an apparent sub-population that failed to exhibit postnatal weight gain, had muscles of equivalent ultrastructural composition to those of their heavier littermates. This indicates that the muscles of low birthweight pigs develop, and appear capable of functioning physiologically and biochemically as well as those of heavier birthweight pigs; the quality of individual myofibres with respect to subcellular structure is evidently unimpaired by growth restriction *in utero*.

SUMMARY

An ultrastructural investigation of the postnatal development of oxidative and non-oxidative fibres from the deep and superficial portions of the semitendinosus muscle, respectively, was undertaken on 32 pure bred Large White pigs from a total of 11 litters. This study quantifies the changes in mitochondrial, lipid droplet and myofibrillar content of these two myofibre types between birth and 84 days of age, and evaluates differences between the largest male (mean birthweight of 1559 g), smallest normal male (1147 g), and runt (758 g) littermates. The oxidative and

non-oxidative fibres, as well as possessing different complements of mitochondria, lipid droplets and myofibrils, showed different rates of myofibrillar accumulation. The relatively small postnatal change in the percentage volume of myofibrils of oxidative fibres, as opposed to the high change within the non-oxidative fibres, presented a cytological basis by which to explain the differential effects of growth retardation on these fibre types. The ultrastructural composition of myofibres was not impaired by reduced birthweight except when, as in two extreme cases, birthweight was severely reduced. In these instances the myofibrillar percentage volume of the non-oxidative fibres was greatly affected.

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REFERENCES

ASHMORE, C. R. & SUMMERS, P. J. (1981). Stretch induced growth in chicken wing muscles; myofibrillar proliferation. *American Journal of Physiology* **241**, C93–97.

BEDI, K. S., BIRZGALIS, A. R., MAHON, M., SMART, J. L. & WAREHAM, A. C. (1982). Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and adult refed animals. *British Journal of Nutrition* **47**, 417–431.

BRADLEY, R., WARD, P. S. & BAILEY, J. (1980). The ultrastructural morphology of the skeletal muscles of normal pigs and pigs with Splayleg from birth to one week of age. *Journal of Comparative Pathology* **90**, 433–446.

DEUTSCH, K. & DONE, J. T. (1971). Congenital myofibrillar hypoplasia of piglets: ultrastructure of affected fibres. *Research in Veterinary Science* **12**, 176–177.

EISENBERG, B. R. & KUDA, A. M. (1976). Discrimination between fibre populations in mammalian skeletal muscle by using ultrastructural parameters. *Journal of Ultrastructure Research* **54**, 76–88.

EISENBERG, B. R., KUDA, A. M. & PETERS, J. (1974). Stereological analysis of mammalian skeletal muscle. *Journal of Cell Biology* **60**, 732–754.

EISENBERG, B. R. & SALMONS, S. (1981). The reorganisation of subcellular structure in muscle undergoing fast-to-slow type transformation. *Cell and Tissue Research* **220**, 449–471.

ELIAS, H. & HYDE, D. M. (1980). An elementary introduction to stereology (quantitative microscopy). *American Journal of Anatomy* **159**, 412–446.

GAUTHIER, G. F. (1968). The relationship of ultrastructure and cytochemical features to colour in mammalian skeletal muscles. *Anatomical Record* **160**, 352.

GAUTHIER, G. F. & PADYKULA, H. A. (1966). Cytological studies of fibre types in skeletal muscle. A comparative study of the mammalian diaphragm. *Journal of Cell Biology* **28**, 333–354.

GOLDSPINK, G. (1965). Cytological basis of decrease in muscle strength during starvation. *American Journal of Physiology* **209**, 100–104.

GOLDSPINK, G. (1970). The proliferation of myofibrils during muscle fibre growth. *Journal of Cell Science* **6**, 593–603.

GOTTSCHALL, J. M. (1980). Electron microscopical characterisation of muscle fibre types of the rat diaphragm using a cluster analysis. *Anatomischer Anzeiger* **147**, 168–179.

HEGARTY, P. V. J. & ALLEN, C. E. (1978). Effect of pre-natal runting on the post-natal development of skeletal muscles in swine and rats. *Journal of Animal Science* **46**, 1634–1640.

HELANDER, E. & THULIN, C. A. (1962). Isometric tension and myofilament cross-sectional area in striated muscle. *American Journal of Physiology* **202**, 824–826.

KRAELING, R. R., RAMPACEK, G. B., CAMPION, D. R. & RICHARDSON, R. L. (1978). Longissimus muscle and plasma enzymes and metabolites in foetally decapitated pigs. *Growth* **42**, 457–468.

KRIEGER, D. A., TATE, C. A., MCMILLIN-WOOD, J. & BOOTH, F. W. (1980). Populations of rat skeletal muscle mitochondria after exercise and immobilisation. *Journal of Applied Physiology* **48**, 23–28.

MACKIE, B. G. & TERJUNG, R. L. (1983). Blood flow to different skeletal muscle fibre types during contraction. *American Journal of Physiology* **245**, H265–275.

MCMEEKAN, C. P. (1940). Growth and development in the pig with special reference to carcass quality characteristics. I. *Journal of Agricultural Science* **30**, 276–344.

MOODY, W. G., ENSER, M. B., WOOD, J. D., RESTALL, D. J. & LISTER, D. (1978). Comparison of fat and muscle development in Pietrain and Large White piglets. *Journal of Animal Science* **46**, 618–633.

NINOMIYA, J. G., ECHEVERRÍA, O. M. & VÁZQUEZ-NIN, G. H. (1981). Morphological studies of fibre types of striated muscle fibres of the Cremaster in the guinea pig. *Acta anatomica* **111**, 240–246.

OLDFORS, A., MAIR, G. P. & SOURANDER, P. (1983). Muscle changes in protein deprived young rats. A morphometrical, histochemical and ultrastructural study. *Journal of Neurological Science* **59**, 291–302.

PADYKULA, H. A. & GAUTHIER, G. F. (1970). The ultrastructure of the neuromuscular junctions of mammalian red, white and intermediate skeletal muscle fibres. *Journal of Cell Biology* **46**, 27–41.

POWELL, S. E. & ABERLE, E. D. (1980). Effects of birthweight on growth and carcass composition of swine. *Journal of Animal Science* **50**, 860–868.

SALMONS, S., GALE, D. R. & SRÉTER, F. A. (1978). Ultrastructural aspects of the transformation of muscle fibre type by long term stimulation: changes in Z discs and mitochondria. *Journal of Anatomy* **127**, 17–31.

SJÖSTRÖM, M., KIDMAN, S., HENRIKSSON LARSÉN, K. & ÄNGQUIST, K. (1982). Z- and M-band appearance in different histochemically defined types of human skeletal muscle fibres. *Journal of Histochemistry and Cytochemistry* **30**, 1–11.

SUZUKI, A. (1974). Histochemical study of β-hydroxybutyrate dehydrogenase activity in skeletal muscle fibres in normal and starved sheep. *Japanese Journal of Zootechnological Science* **45**, 401–407.

THURLEY, D. C., GILBERT, F. R. & DONE, J. T. (1967). Congenital Splayleg of piglets: myofibrillar hypoplasia. *Veterinary Record* **80**, 302–304.

WARD, P. S. & BRADLEY, R. (1980). The light microscopical morphology of the skeletal muscles of normal pigs and pigs with Splayleg from birth to one week of age. *Journal of Comparative Pathology* **90**, 421–431.

WEIBEL, E. R., KISTLER, G. S. & SCHERLE, W. F. (1966). Practical stereological methods for morphometric cytology. *Journal of Cell Biology* **30**, 23–38.

WEIBEL, E. R. (1972). A stereological method for estimating volume and surface of sarcoplasmic reticulum. *Journal of Microscopy* **95**, 229–242.

Anim. Prod. 1988, **47**: 291-295

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CATCH-UP GROWTH IN PIGS: A RELATIONSHIP WITH MUSCLE CELLULARITY

S. E. HANDEL† AND N. C. STICKLAND
Department of Anatomy, The Royal Veterinary College, Royal College Street, London NW1 0TU

ABSTRACT

An investigation was made into a possible relationship between the relative growth potential and skeletal muscle fibre number in low birth weight Large White pigs and their heaviest birth-weight littermates. Estimates of the total fibre number of *m. semitendinosus* were made from 23 pairs of 'small' and 'large' littermates. Littermates were slaughtered at the same age and were of a mean live weight 86 (s.d. 15) kg. Small littermates (mean birth weight 939 (s.d. 164) g) had, on average, lower live weights at slaughter ($P < 0{\cdot}001$) and lower muscle fibre numbers ($P < 0{\cdot}05$) than their large littermates (mean birth weight 2085 (s.d. 343) g). However, pigs within the low birth-weight category exhibited various degrees of catch-up growth with their heaviest littermates and some individuals actually exceeded their large littermates in live weight at slaughter. The relative live weights at slaughter and relative muscle fibre numbers of small and large littermates were determined by expressing the value for the small littermate as a proportion of the corresponding value for its large littermate. No correlation existed between relative live weight at slaughter and relative muscle fibre number of small and large littermates. The small littermates were then divided into two groups by their mean value of relative small to large slaughter weight (0·84). The littermates of low relative slaughter weight ($\leq 0{\cdot}84$) exhibited a large range of muscle fibre numbers although their mean fibre numbers were lower than their large littermates ($P < 0{\cdot}05$). This large range was probably responsible for the lack of correlation mentioned above. Those small littermates which exhibited a good degree of catch-up growth (relative slaughter weight $>0{\cdot}84$) possessed fibre numbers which were not significantly different from those of their large littermates. These results suggest that pigs which exhibit an appreciable degree of catch-up growth always contain high relative numbers of fibres in their muscles. Those which show a poor degree of catch-up growth ($\leq 0{\cdot}84$) exhibit a range of fibre numbers because there may be environmental and other factors which prevent some pigs from realizing their full growth potential. The results of the present study advocate muscle fibre number as an indicator at birth of potential for post-natal growth performance.

INTRODUCTION

SKELETAL muscle constitutes a major component of the carcass in commercial meat-producing animals. Growth potential of muscle in these animals is limited post natally by hypertrophy of its component muscle fibres. Assuming that the amount of hypertrophy is mainly governed by the maximum size at which the fibres are capable of functioning efficiently, then the size to which the muscle can grow is largely determined with the completion of hyperplasia. The growth potential of muscle and therefore, to a large extent, that of the whole body, is determined *in utero* (when muscle fibre hyperplasia ceases).

Low birth weight in the pig is known to be associated with the acquisition of a reduced skeletal muscle fibre number *in utero* (Wigmore and Stickland, 1983), an effect maintained post natally (Handel and Stickland, 1984) and probably common to all skeletal muscles in the body (Stickland and Goldspink, 1973). In an investigation of the effects of low birth weight on muscle characteristics after birth it was discovered that although low birth weight is associated with a reduced total muscle fibre number ($P < 0{\cdot}001$; Handel and Stickland, 1984) this effect is not consistent. During the course of this study it was also noted that low birth-weight littermates are capable of exhibiting

† Present address: Department of Human Anatomy and Cell Biology, The University of Liverpool, PO Box 147, Liverpool L69 3BX.

'catch-up' growth by which they attained live weights equivalent to, or approaching, those of their large littermates although, on average, they maintained lower live weights ($P < 0{\cdot}001$).

In view of these findings it was considered valuable to investigate a possible relationship between low live-weight gain and reduced total skeletal muscle fibre number of low birth-weight littermates relative to their heavier siblings. Likewise, it was important to establish whether some low birth-weight pigs possessed any potential for catch-up growth in terms of relatively high fibre numbers. It was therefore proposed to test the hypothesis that low birth-weight pigs which exhibit an appreciable degree of catch-up growth contain muscle fibre number values similar to their large littermates. Pigs which do not catch up to any great extent could exhibit a range of fibre number values as there may be various environmental factors which prevent the full realization of the growth potential indicated by muscle fibre number content.

MATERIAL AND METHODS

Selection of animals

The animals investigated in this study comprised 40 Large White pigs selected by their relative weights at birth. At birth the heaviest littermate (defined as 'large' littermate) was selected together with any siblings of half, or less, the weight of their large littermate (defined as 'small' littermate). Littermates were of either sex; the sex of the pig does not appear to influence total muscle fibre number (Stickland, 1973). Due to the occasional existence of two equally heavy large littermates or more than one small littermate there were 23 resultant pairs of littermates. Littermates were slaughtered at the same age when they had both reached a sufficient commercial slaughter weight.

Preparation of muscle samples

The *m. semitendinosus* was removed from the left side of each carcass immediately after slaughter at a commercial abattoir and placed on ice. A complete transverse slice, of 2 to 3 mm thickness, was taken from the mid belly of the muscle on returning to the laboratory. The transverse sectional area of the muscle mid belly was recorded. The large size of the mid-belly slice necessitated the preparation of one sample from each of the deep and superficial portions of the muscle slice. The muscle samples were mounted with water on pieces of 5-mm cork sheeting placed on a cryostat chuck and rapidly frozen in dichlorodifluoromethane (Arcton 12, ICI Ltd), cooled to its melting point of −158°C with liquid nitrogen. Serial sections (10 μm), cut on a Bright retracting rotary cryostat at −22°C, were picked up on coverslips and allowed to thaw and dry out at room temperature for 1·5 h. Muscle sections were then stained for the histochemical demonstration of alkaline pre-incubated adenosine triphosphatase (Guth and Samaha, 1970). The histochemical stain clearly differentiates fibres due to the range of staining intensities demonstrated and is therefore ideal for fibre counting.

The number of muscle fibres in a total of 15 randomly selected areas from both the deep and superficial portions were counted and estimates of total muscle fibre number made using these fibre counts from frozen muscle sections together with the whole fresh mid-belly transverse sectional area determination (which was shown by Gunn (1976) not to be affected by freezing). The mean number of fibres counted in the sample areas amounted to a total of 11150 (s.d. 1859) per animal which represented proportionately 0·022 of the estimated total fibre number for *m. semitendinosus*.

Paired observations for littermates were analysed to test the significance of differences between mean values (paired *t* test) of the histological data for groups of animals. This method of analysis was chosen to eliminate the level of variation in muscle fibre number caused by between-litter differences (Handel, 1984), as opposed to that within-litters which is mainly attributed to birth weight differences between littermates.

RESULTS

The mean birth weights of the large and small littermates were 2085 (s.d. 343) g and 939 (s.d. 164) g, respectively. The small

littermates were, on average, proportionately 0·45 the weight of their large littermates at birth ($P < 0·001$). The mean slaughter weight was 86 (s.d. 15) kg and the mean age of slaughter was 157 (s.d. 12) days.

The live weights at slaughter and total *m. semitendinosus* fibre number results for the littermates are displayed in Table 1. The mean relative live weight of the small to large littermates at slaughter, a value of 0·84, indicated that the small littermates weighed, on average, less than their heavier littermates ($P < 0·001$) although the relative values ranged from 0·53 to 1·07. The 0·91 mean relative total muscle fibre number of the small to large littermates suggested that the myofibre number was less ($P < 0·05$) in the small relative to large littermates although the values ranged above 1·00 (from 0·58 to 1·25), as for relative live weight.

No significant correlation existed between the relative degree of catch-up growth evident between small and large littermates at slaughter and their relative total muscle fibre numbers. However, not all small littermates with high fibre numbers would be expected to realize their full growth potential for various reasons including environmental factors. On the other hand, all small littermates which did exhibit good catch-up growth would be expected to have a high fibre number. In order to test this hypothesis, the pigs were divided into two groups by the mean value of the proportion of small to large slaughter weights (0·84; see Table 1). The group which exhibited lower catch-up growth (i.e. proportion of small to large weight at slaughter was $\leq 0·84$) contained significantly fewer muscle fibres than their large littermates ($P < 0·05$). The group which exhibited a good degree of catch-up growth (i.e. proportion of small to large slaughter weight was $>0·84$) did not differ significantly in fibre number content from their large littermates. The relative fibre numbers of small to large littermates ranged from 0·58 to 1·12 and from 0·80 to 1·25 in the $\leq 0·84$ and $>0·84$ groups, respectively.

DISCUSSION

Animals that are lighter than average at birth are not necessarily destined to become small adults. If prenatally growth-retarded littermates have the potential to grow well and they are able to realize that potential they can attain live weights equivalent to those of their heavier birth-weight siblings, as shown by low birth-weight littermates in the

TABLE 1

Live weight at slaughter and total muscle fibre number data for large and small littermates

	Large littermates		Small littermates		Mean value of small littermates as a proportion of large and significance of difference
	Mean	s.d.	Mean	s.d.	
Total group studied (no. = 23)					
Live weight (kg)	92	9	77	17	0·84***
Fibre number	538735	75339	488852	66418	0·91*
≤0·84 group (no. = 12)					
Live weight (kg)	90	7	66	11	0·73***
Fibre number	548851	79958	456871	60811	0·83*
>0·84 group (no. = 11)					
Live weight (kg)	95	9	89	14	0·94*
Fibre number	527698	72095	523740	55416	0·99

present, and those of an earlier (Handel, 1984) study.

The fact that an animal does not necessarily realize its growth potential probably accounts for the lack of correlation between relative live weight at slaughter and muscle fibre number between littermates of the present study. If it is the case that animals with a low relative live weight do not inevitably possess low muscle fibre numbers, through their lack of expression of growth potential (muscle fibre number being an indicator of this potential), but those with a relatively high live weight do correspondingly possess high relative muscle fibre numbers, it would suggest that muscle fibre number is indeed an indicator of growth potential. To investigate this phenomenon (as stated in the RESULTS) the mean relative small to large littermate live weight of 0·84 was chosen to divide small littermates presenting a relatively low degree of catch-up growth ($\leq$0·84 group) from those presenting an appreciable (above average) degree of catch-up growth (>0·84 group). The results of the investigation (Table 1) showed that the small littermates of the $\leq$0·84 group had significantly lower ($P < 0{\cdot}05$) muscle fibre numbers than their large littermates while the small littermates of the >0·84 group had muscle fibre numbers essentially equal to those of their large littermates. This appears to verify the hypothesis that for small birth-weight littermates to achieve live weights approaching those of their large littermates they must have a similar muscle fibre number; in this investigation it was in the order of 0·99 (s.d. 0·14) of the large littermate fibre number. The range in relative small to large littermate muscle fibre number of the $\leq$0·84 group reached as high as 1·12 (for complete range see RESULTS). This suggested that there were small siblings in this group that may have been capable of expressing a greater degree of catch-up growth given a greater length of time and adequate nutrition.

Small size at birth will inevitably impose a physical disability on a littermate which can have adverse consequences on its growth and development. Milk consumption is the most important factor affecting weight gain in the young pig (Lodge and McDonald, 1959; Lucas, 1968), and pigs of low birth weight compete less successfully for food, especially during suckling (Hartsock and Graves, 1970; England, 1974; Hemsworth, Winfield and Mullaney, 1976). Powell and Aberle (1980) reduced the level of competition during pre-weaning growth by cross-fostering pigs to create small groups of similar sized, suckling animals. Despite these expediences, pigs of low birth weight failed to grow as fast or utilize food for weight gain as efficiently as their heavier birth-weight littermates. The general lack of success gained by reducing the level of behavioural competition between littermates during suckling on development to 96 or 109 kg (Powell and Aberle, 1980) could be explained by the fact that these animals had a restricted growth capacity as indicated by a reduced skeletal muscle hyperplasia *in utero* (Powell and Aberle, 1981), so the steps that were taken to promote growth were wasted.

It would be of interest to know at birth whether an animal of low relative weight had the potential for catch-up growth and would, therefore, benefit from suckling in small groups. An indicator muscle such as the *m. flexor digiti V brevis* proposed, by Stickland and Goldspink (1973), as representative of muscle fibre content and growth characteristics would be of tremendous value in selecting low birth weight littermates with the potential for catch-up growth (i.e. a relatively high muscle fibre number, e.g. 0·99 (s.d. 0·14) that of the largest littermate). These animals would probably have the ability, under a regime allowing adequate food intake from birth, to express their growth potential and reach weights at slaughter equivalent to those of their littermates of heaviest birth weight.

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REFERENCES

ENGLAND, D. C. 1974. Husbandry components in prenatal and perinatal development in swine. *Journal of Animal Science* **38:** 1045-1049.

GUNN, H. M. 1976. The effect of freezing on the transverse sectional area of a muscle. *Histochemical Journal* **8:** 651-652.

GUTH, I. and SAMAHA, F. J. 1970. Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28:** 365-367.

HANDEL, S. E. 1984. Effects of low birthweight on postnatal development of skeletal muscle in the pig. *Ph.D. Thesis, University of Edinburgh.*

HANDEL, S. E. and STICKLAND, N. C. 1984. Muscle cellularity and its relationship with birth weight. *Journal of Anatomy* **139:** 726 (Abstr.).

HARTSOCK, T. G. and GRAVES, H. B. 1970. Ontogeny of the nursing order in newborn pigs. *Journal of Animal Science* **31:** 174 (Abstr.).

HEMSWORTH, P. H., WINFIELD, C. G. and MULLANEY, P. D. 1976. Within-litter variation in the performance of piglets to three weeks of age. *Animal Production* **22:** 351-357.

LODGE, G. A. and MCDONALD, I. 1959. The relative influence of birth weight, milk consumption and supplementary food consumption upon the growth rates of suckling pigs. *Animal Production* **1:** 139-144.

LUCAS, I. A. M. 1968. Practical implications of some genetic and environmental influences on growth and development in pigs. In *Growth and Development of Mammals* (ed. G. A. Lodge and G. E. Lamming), pp. 466-497. Butterworths, London.

POWELL, S. E. and ABERLE, E. D. 1980. Effects of birth weight on growth and carcass composition of swine. *Journal of Animal Science,* **50:** 860-868.

POWELL, S. E. and ABERLE, E. D. 1981. Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. *Journal of Animal Science* **52:** 748-756.

STICKLAND, N. C. 1973. The growth and development of skeletal muscles in pigs. *Ph.D. Thesis, University of Hull.*

STICKLAND, N. C. and GOLDSPINK, G. 1973. A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16:** 135-146.

WIGMORE, P. M. C. and STICKLAND, N. C. 1983. Muscle development in large and small pig foetuses. *Journal of Anatomy* **137:** 235-245.

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Cattle at Risk for Dark-Cutting Beef Have a Higher Proportion of Oxidative Muscle Fibres

A. C. Zerouala & N. C. Stickland

Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, UK

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ABSTRACT

The aim of this investigation was to compare muscle fibre type characteristics in dark-cutting (DFD) and normal beef animals. Three categories of animal were used in this investigation: 9 DFD bulls, 10 DFD steers and 10 normal bulls. For each animal, frozen sections of longissimus dorsi *muscle were stained histochemically. The results showed that there were proportionately more slow, oxidative (SO) fibres in DFD bulls and DFD steers than in normal bulls. As far as fast, glycolytic (FG) fibres were concerned DFD bulls contained fewer of this type than DFD steers and normal bulls. When the results on fibre proportions were combined with those on mean fibre cross-sectional areas it was found that both DFD groups contained a smaller proportion of muscle exhibiting FG characteristics than normal bulls. Furthermore, the combination of all oxidative fibres, i.e. SO and fast, oxidative, glycolytic (FOG) fibres, showed that, both by numbers and relative areas, the two DFD groups (especially DFD bulls) exhibited significantly more oxidative metabolism in the* longissimus dorsi *muscle than the normal animals.*

INTRODUCTION

Dark-cutting beef or DFD (dark, firm, dry) is an identifiable problem with incidence rates ranging from 1–5% for steers and heifers, 6–10% for cows and 11–15% for young bulls. Estimates for loss in carcass value due to dark-cutting problems range up to 20% with an average of 10% (Tarrant, 1980).

The term dark-cutting beef is applied to beef which exhibits a dark, sometimes a blackish, colour when cut and fails to develop the 'cherry red' colour expected by the meat trade. The basis for dark cutting beef is apparently activation of muscle glycolytic metabolism culminating in low muscle glycogen stores at the time of slaughter and therefore, leading to a high ultimate pH (Hedrick *et al.*, 1959). It is now generally accepted that stress is associated with this glycogen depletion and is a significant contributor to the problem (Lawrie, 1958; Grandin, 1978, 1980; Tarrant, 1980) but little is known about why some cattle, of the same category and kept under similar conditions, are affected and not others.

In pigs, the pale, soft and exudative condition (PSE), which is also stress-related, has been shown to be associated with a relatively high proportion of fast glycolytic fibres which may lead to a rapid pH fall at slaughter (Ashmore, 1974). It might be expected that if a high proportion of glycolytic fibres causes a rapid pH fall at slaughter then a low proportion of these fibres may restrict pH fall *post mortem*. However, little information on the cellular basis of DFD is available. The hypothesis suggested here is that cattle exhibiting a relatively high level of oxidative metabolism (i.e. low level of glycolytic metabolism) within their muscles are more susceptible to the development of DFD. This hypothesis was tested by comparing fibre type proportions in dark-cutting and normal animals which had been slaughtered under normal abattoir conditions.

MATERIALS AND METHODS

Choice of muscle

Longissimus dorsi muscle was selected for this investigation for many reasons including the fact that it is of commercial importance; it is most affected in DFD (Tarrant, 1980); it is observed at an early stage at quartering; it is much studied by other workers; and it is a readily accessible muscle for sampling (Tarrant & Sherington, 1980).

Selection of animals

Animals used were Friesian yearling bulls and steers which had been barley fed indoors in the period leading up to slaughter. The animals originated from various regions within the UK. Three groups of animals were selected in this study: a group of 9 DFD bulls, a group of 10 DFD steers and a group of 10 normal bulls.

The mean carcass weight did not differ between the three groups; the overall mean was 270·9 kg. The animals were selected over a period

extending from January to May. At the abattoir the animals from which samples were taken were stunned with a captive-bolt pistol and exsanguinated according to normal abattoir procedure. Selection of dark and normal carcasses was based upon pH measurement and colour assessment at 48 h *post mortem*. Three readings of pH were taken across the cut surface of the *longissimus dorsi* muscle and the mean of these values taken. The pH meter used was the portable type (PI 8070; Portec Instrumentation Ltd, Ampthill, Bedford) with a combined glass electrode. Measurement of carcass pH was made by inserting the electrode directly into the muscle. The colour was assessed by a qualified veterinarian and meat inspectors at the abattoir. Carcasses with a pH above 6 were considered dark cutters and below this figure as normal. All dark cutters selected in this way exhibited slightly dark to very dark meat colour in contrast to the normal carcasses which exhibited a bright colour.

Sampling

After selecting dark and normal carcasses, samples for histochemistry were taken. The samples were approximately 1 cm^3 in size and were taken from the *longissimus dorsi* muscle at the eleventh rib level from carcasses hanging in the chillers (4°C) at 48 h *post mortem*. All the samples were taken from the same region and were orientated on the cryostat for transverse sections. In pilot studies it was found that there was no significant difference in muscle fibre type proportions between 9 sample sites in the *longissimus dorsi* at the eleventh rib level. In any event all samples were removed from the same region, i.e. the central one. Another pilot study showed no significant difference in detection and proportion of fibre types using myosin adenosine triphosphatase (ATPase) reactions on muscle obtained at 1 h, 24 h and 48 h *post mortem*. Over this time period there is a considerable change in pH from approximately 6·7 at 1 h to 5·5 at 48 h *post mortem* in normal carcasses. This study also demonstrated therefore, that a change in muscle pH does not affect the results on fibre type proportions. Serial frozen sections of all samples were taken and reacted for both alkaline and acid myosin ATPase (Guth & Samaha, 1970), succinic dehydrogenase (SDHase) (Nachlas *et al.*, 1957) and glycogen phosphorylase (GPase) (Takeuchi, 1956). Using these techniques three main fibre types could be distinguished: slow oxidative (SO), fast oxidative glycolytic (FOG) and fast glycolytic (FG).

Determination of proportions and sizes of the different fibre types

For each muscle sample, three randomly selected areas were viewed using a VID II image analysis system (Analytical Measuring Systems Ltd, Essex)

connected to a light microscope. The three fibre types could be distinguished using the alkaline ATPase sections (three different colours could be distinguished) but classifications were confirmed by reference to the SDHase, GPase and acid ATPase sections. The proportions and mean cross-sectional areas of each fibre type were determined using the image analysis system and were based on a total of approximately 200 fibres for each sample. Differences between the three groups of animals were assessed by analysis of variance followed by Newman-Keul's test.

RESULTS

The results are summarised in Tables 1 to 3 and Fig. 1. Table 1 shows that there were proportionately more SO fibres in DFD bulls and DFD steers than in normal bulls. As far as FG fibres were concerned DFD bulls contained fewer of this type than the other two groups. When the results from Table 1 (on fibre proportions) were combined with Table 2 (on mean fibre cross-sectional areas) it was found that both DFD groups contained a smaller proportion of muscle exhibiting FG characteristics than normal bulls (Table 3). It has been shown for many animals (e.g. mouse: Goldspink & Ward, 1979) that FOG fibres exhibit strong oxidative capacity (more so than SO fibres by SDHase activity) and only a weak glycolytic capacity

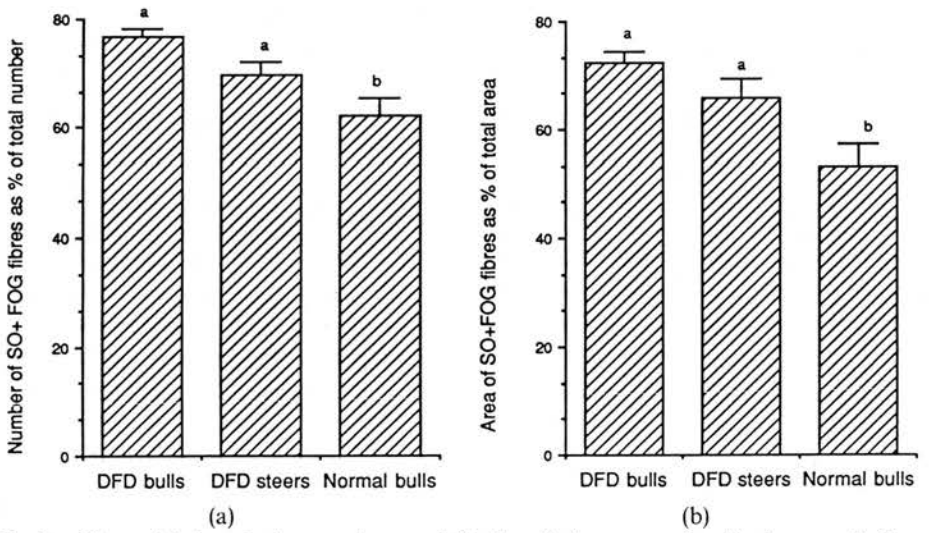

Fig. 1. Shows (a) the relative numbers and (b) the relative areas of oxidative muscle fibres (SO + FOG) in the *longissimus dorsi* muscle of the three groups of animals. Bars represent standard errors of the means. Groups with different letters are significantly ($P < 0{\cdot}05$) different as assessed by the Newman-Keul test.

TABLE 1
Mean Proportions of Fibre Types in *Longissimus dorsi* Muscle for the Three Categories of Animal (Means ± SEM)

	SO	*FOG*	*FG*
DFD bulls	$27{\cdot}5 \pm 1{\cdot}4^a$	$49{\cdot}0 \pm 1{\cdot}5^a$	$23{\cdot}5 \pm 1{\cdot}5^a$
DFD steers	$27{\cdot}0 \pm 1{\cdot}9^a$	$41{\cdot}5 \pm 2{\cdot}3^a$	$31{\cdot}5 \pm 2{\cdot}5^b$
Normal bulls	$20{\cdot}8 \pm 1{\cdot}8^b$	$41{\cdot}5 \pm 2{\cdot}7^a$	$37{\cdot}7 \pm 3{\cdot}1^b$

SO = Slow oxidative fibres.
FOG = Fast oxidative glycolytic fibres.
FG = Fast glycolytic fibres.
SEM = Standard of error of the mean.
Within each column values with different superscripts are significantly different at $P < 0{\cdot}05$.

TABLE 2
Cross-Sectional Area (μm^2) of Fibre Types in the *Longissimus dorsi* Muscle for the Three Categories of Animal (Means ± SEM)

	SO	*FOG*	*FG*
DFD bulls	$2\,244 \pm 207^a$	$3\,821 \pm 385^a$	$4\,035 \pm 393^a$
DFD steers	$2\,590 \pm 243^a$	$4\,404 \pm 427^a$	$5\,448 \pm 458^b$
Normal bulls	$2\,090 \pm 232^a$	$3\,004 \pm 286^b$	$4\,032 \pm 436^b$

SEM = Standard of error of the mean.
Within each column values with different superscripts are significantly different at $P < 0{\cdot}05$.

TABLE 3
Percentage Cross-Sectional Area of the *Longissimus dorsi* Muscle Occupied by Each Fibre Type for the Three Categories of Animal (Means ± SEM)

	SO	*FOG*	*FG*
DFD bulls	$17{\cdot}9 \pm 0{\cdot}9^a$	$54{\cdot}4 \pm 1{\cdot}8^a$	$27{\cdot}7 \pm 2{\cdot}0^a$
DFD steers	$19{\cdot}4 \pm 2{\cdot}7^a$	$44{\cdot}8 \pm 2{\cdot}9^b$	$35{\cdot}8 \pm 3{\cdot}6^a$
Normal bulls	$13{\cdot}9 \pm 2{\cdot}7^a$	$39{\cdot}3 \pm 3{\cdot}4^b$	$46{\cdot}8 \pm 4{\cdot}1^b$

SEM = Standard of error of the mean.
Within each column values with different superscripts are significantly different at $P < 0{\cdot}05$.

(weaker than FG fibres by GPase activity). In this study also, examination of the relative staining intensities of the different fibre types revealed that FOG fibres exhibited strong SDHase activity (relative to SO fibres) and weak GPase activity (relative to FG fibres). it is, therefore, more appropriate to combine all oxidative fibres (SO and FOG) rather than glycolytic fibres in any attempt to assess relative metabolic activities of the different samples. This was carried out to produce Fig. 1 which shows that, both by numbers and relative areas, the two DFD groups exhibited significantly more oxidative metabolism in the *longissimus dorsi* muscle than the normal animals.

DISCUSSION

Animals exhibiting dark-cutting 48 h *post mortem* had a significantly greater proportion of oxidative fibres in *longissimus dorsi* than normal animals. Furthermore, DFD bulls contained fewer and smaller FG fibres than the DFD steers. Taken as a whole, the results clearly demonstrated a shift to more oxidative metabolism in the muscle of DFD cattle with some evidence for a greater shift in DFD bulls. The following hypothesis is, therefore, confirmed from our results: that cattle exhibiting a relatively high level of oxidative metabolism within their muscles are more susceptible to the development of DFD.

In order to understand the relationship between stress and the pattern of glycogen depletion in different muscle fibres, dark cutting has been induced by adrenaline injection (Hedrick *et al.*, 1959; Ashmore *et al.*, 1973; Lacourt & Tarrant, 1985) or 'mixing' stress (Lacourt & Tarrant, 1985). The results demonstrated that glycogen is selectively depleted in muscle fibres in response to stress. In fact, Ashmore (1974) reported an unpublished observation that, in response to epinephrine injection, glycogen is depleted more rapidly from α Red fibres than α White fibres. He suggested that this pattern of glycogen depletion was a result of the fact that α Red (FOG) fibres have a more substantial blood supply than αWhite (FG) fibres and therefore, α Red fibres would respond to adrenaline at a faster rate. These reports support our hypothesis that dark-cutting beef is closely related to a high proportion of oxidative fibre types within muscles. In other words, it is the ratio of SO and FOG fibres to FG fibres which is important. It is now widely accepted that stress is an important factor in the development of dark-cutting beef but most experimental work on dark-cutting beef has involved animals which have been highly stressed, often by adrenaline injection. The form of stress used is such that all experimental animals

deplete their glycogen stores, leading ultimately to a high post-mortem pH. The importance of the present investigation, however, is that it has demonstrated a probable reason for some animals being more susceptible to development of DFD than others under non-experimental abattoir stress situations.

An important question which arises from these results concerns the factors which affect fibre type proportions in bovine skeletal muscle. It is interesting that many of the factors which are known to affect fibre type proportions in laboratory studies (exercise, sex, weight, temperature) may be linked to some of the suggestions put forward as contributory to DFD problems in a survey by Tarrant (1980). Breed is one of these factors and it is clear from muscle histochemical studies that the genetic background of an animal has a marked effect on fibre type proportions (Ashmore, 1974). The suggestion of a seasonal variation in DFD incidence may be linked to the fact that temperature has been shown to affect the proportion of oxidative fibres (Dauncey, 1988). Handel and Stickland (1987) showed that heavier pigs contain a relatively greater proportional area of slow fibres than lighter animals and this may be one reason why heavier animals tend to exhibit a higher indicence of DFD. Bulls are said to be affected by DFD more than steers and there is some indication here that DFD steers are intermediate in their muscle fibre characteristics between DFD bulls and normal bulls (Fig. 1). Taken as a whole it would seem that all suggestions put forward in Tarrant's survey (1980) as contributory factors to the problem of DFD may be relevant insofar as they may all relate to an effect on fibre type characteristics.

Although stress is clearly the trigger which may induce dark cutting it is apparent from this investigation that the factor which determines the susceptibility of animals to this trigger is the relative dependence on a glycolytic or oxidative metabolism within muscles. In the short term a reduction in stress will reduce DFD problems but in the long term care must be taken that genetic selection for beef animals does not unwittingly select for an increase in oxidative metabolism (i.e. an increase in the relative proportions of SO and FOG fibres).

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REFERENCES

Ashmore, C. R., Carroll, F. & Doerr, L. (1973). *J. Anim. Sci.*, **2**, 435.
Ashmore, C. R. (1974). *J. Anim. Sci.*, **5**, 1158.
Dauncey, M. J. (1988). *Eur. J. Appl. Physiol.*, **58**, 239.
Goldspink, G. & Ward, P. S. (1979). *J. Physiol.*, **296**, 453.
Grandin, T. (1978). *J. Anim. Sci.*, **47**, Suppl. 1, 217.
Grandin T. (1980). *Int. J. Stud. Anim. Prob.*, **5**, 313.
Guth, L. & Samaha, F. J. (1970). *Exp. Neurol.*, **28**, 365.
Handel, S. E. & Stickland, N. C. (1987). *J. Anat.*, **152**, 107.
Hedrick, H. B., Biollot, J. B., Brady, D. E. & Naumann, H. D. (1959). *Etiology of dark cutting beef.* Mo. Agri. Exp. Sta. Res. Bull., 717.
Lacourt, A. & Tarrant, P. V. (1985). *Meat Sci.*, **15**, 85.
Lawrie, R. A. (1958). *J. Sci. Food Agric.*, **9**, 721.
Nachlas, M. M., Tsou, M., De Souza, E., Cheng, C. & Selimgman, A. M. (1957). *J. Histochem. Cytochem.*, **5**, 420.
Takeuchi, T. (1956). *J. Histochem. Cytochem.*, **4**, 84.
Tarrant, P. V. (1980). In *The problem of dark cutting beef*, ed. D. E. Hood & P. V. Tarrant. Martinus Nijhoff Publishers, The Hague.
Tarrant, P. V. & Sherington, J. (1980). *Meat Sci.*, **4**, 287.

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SOURCES OF VARIATION IN MYOFIBRE NUMBER WITHIN AND BETWEEN LITTERS OF PIGS

C. M. DWYER, AND N. C. STICKLAND
Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU

ABSTRACT

A study of the determinants of inter- and intra-litter variation in muscle fibre number was carried out on five litters of Large White piglets. Fresh frozen, whole mid-belly sections of *m. semitendinosus* were stained to demonstrate acid-stable myosin adenosine triphosphatase activity. From these sections it was possible to identify which fibres had developed as primary and which as secondary fibres. Estimations of total muscle fibre number, total primary fibre number and ratio of secondary fibres to primary fibres were made for each animal. Results demonstrated that primary fibre number varied between litters ($P < 0{\cdot}01$) and was responsible for the variation in total muscle fibre number ($P < 0{\cdot}05$) between litters since there was no significant variation in secondary : primary ratio. Within-litter differences in total fibre number could be attributed to both the secondary : primary ratio and primary fibre number, in almost equal contributions. However, when only the largest and smallest extremes of the litters were compared, variation in fibre number was due to the significant difference in the secondary : primary ratio ($P < 0{\cdot}01$). Taken as a whole, the results appear to show that primary fibre number is responsible for all the variation in muscle fibre number between litters, and also makes a significant contribution, with secondary : primary fibre ratio, to the variation present within a litter. The factors responsible for variations in primary and secondary fibre numbers are discussed.

KEYWORDS: litter traits, myofibrils, pigs.

INTRODUCTION

MUSCLE fibre number is known to be the most important determinant of muscle mass (mouse: Luff and Goldspink, 1979; pig: Miller, Garwood and Judge, 1975). In the pig, muscle fibre hyperplasia is completed during gestation and fibre numbers remain fixed from birth (Staun, 1963; Stickland and Goldspink, 1973). Exercise and nutrition affect size and types of fibres in post-natal animals but fibre number is unaffected (Stickland, Widdowson and Goldspink, 1975; Goldspink and Ward, 1979). However, fibre number can be affected by conditions occurring *in utero* during fibre development, e.g. maternal nutrition (Wigmore and Stickland, 1983; Ward and Stickland, 1991) and innervation (McLennan, 1983). Therefore, pre-natal conditions affecting fibre number can have a long-term effect on post-natal growth rate (Hegarty and Allen, 1978) and size.

Muscle develops in a biphasic manner in the pig (Ashmore, Addis and Doerr, 1973; Swatland, 1973): an initial population of myoblasts fuse rapidly to form primary fibres followed by the slower fusion of a larger population of myoblasts to form secondary fibres. Previous work has shown that variation in muscle fibre number within litters is due to a variation in secondary fibre number; the number of primaries which form is relatively constant for a given litter (Handel and Stickland, 1987). However, this conclusion was based on an examination of only two littermates per litter, selected for extremes of birth weight.

This present investigation was designed to examine the results of Handel and Stickland (1987) in more detail by looking at every member of five litters. The following hypotheses were tested: (i) inter-litter variation is due to variation in primary fibre number, (ii) intra-litter variation is due to variation in

secondary fibre number. A preliminary communication of some of these results has already appeared (Dwyer and Stickland, 1989).

MATERIAL AND METHODS

Five Large White litters, which had been bred and reared under similar conditions, were used in this study. Piglets were weighed each week, to determine growth rate, weaned at 3 weeks and killed at 5 weeks of age by an overdose injection of pentobarbitone sodium into the heart. The *semitendinosus* muscle was dissected out from the left side, weighed and a complete mid-belly slice rapidly frozen in dichlorodifluoromethane (Arcton 12, ICI) cooled to its melting point of –158°C in liquid nitrogen. Sections of 10 μm thickness were cut at –25°C and stained for myosin adenosine triphosphatase activity, using the method of Guth and Samaha (1970).

Mature pigs exhibit a unique arrangement of muscle fibres with central groups of slow fibres surrounded by large numbers of fast fibres. From previous studies (Wigmore and Stickland, 1983) it is known that one of the central slow fibres, in each cluster, was a primary myofibre whereas all others developed as secondary fibres. This therefore means that the number of primary and secondary fibres which formed during development pre-natally can be determined in the post-natal animal.

For each animal (no. = 48) the total cross-sectional area of the muscle was first determined. The number of primaries and total number of fibres were then counted in a number of randomly selected areas to total proportionately about 0·03 to 0·05 of the total number of primaries and fibres present. These data were used to estimate the total number of fibres, the total number of primaries and the ratio of secondary fibres to primary fibres (S : P ratio). A Seescan image analysis machine (Seescan plc, Cambridge, UK) was used throughout.

Inter-litter variations were compared by analysis of variance. Comparisons of largest and smallest littermates were carried out by paired t tests to remove the influence of inter-litter variations. In order to assess the contribution of S : P ratio and primary fibre number on total fibre number within litters correlation coefficients were calculated.

RESULTS

The mean number of muscle fibres for all animals was 358 400 (s.d. = 56 900). However, the distribution was wide with extremes varying by up to 2·5 s.d. from the mean (Figure 1). Muscle fibre number was significantly correlated with muscle weight (r = 0·66, $P < 0·001$) and with average daily increase in weight (ADG) to 5 weeks (r = 0·65, $P < 0·001$; Figure 2). When every animal was considered, regardless of litter, both primary fibre number and S : P ratio were seen to be significantly correlated with total fibre ($P < 0·001$; Figure 3). Correlation coefficients were 0·84 and 0·66 for primary fibre number and S : P ratio respectively.

Inter-litter variations

Analyses of variance were used to compare muscle fibre data between litters. This demonstrated a significant variation in the mean muscle fibre number between litters ($P < 0·05$). This variation appeared to be almost totally due to the significant variations in mean primary fibre number between litters ($P < 0·01$) since the variation in mean S : P ratio was not significant (Figure 4).

Intra-litter variations

Handel and Stickland (1987) concluded that, within litters, variations in S : P ratio were far more responsible for variations in total fibre number than were variations in primary fibre number. However, this was

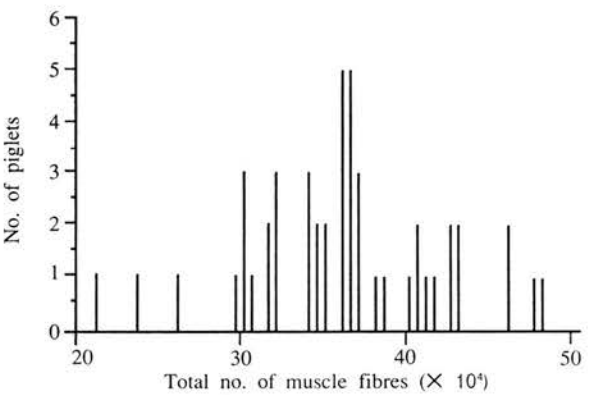

FIG. 1. Distribution of total fibre numbers for all animals (no. = 48).

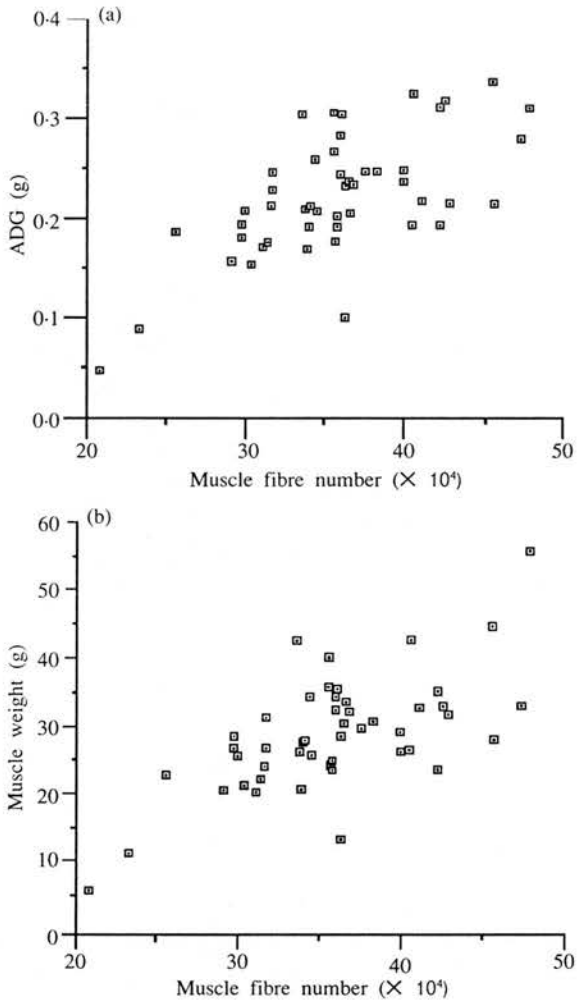

FIG. 2. Correlation of total muscle fibre number with (a) muscle weight; $r = 0{\cdot}66$, $P < 0{\cdot}001$. (b) average daily gain in weight (ADG); $r = 0{\cdot}65$, $P < 0{\cdot}001$.

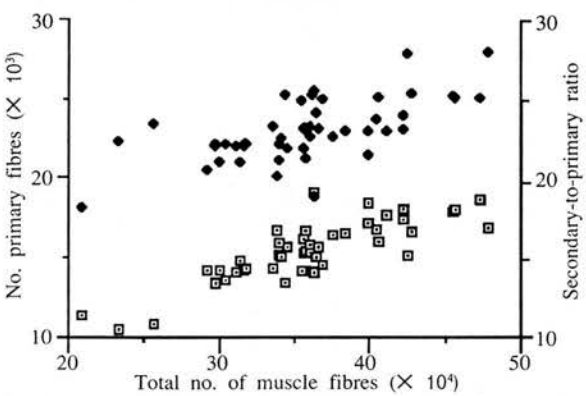

FIG. 3. The correlation of primary fibre number (❑) ($r = 0{\cdot}84$) and S : P ratio (◆) ($r = 0{\cdot}66$) with total number of muscle fibres for all data.

TABLE 1

Litter extremes: paired t *tests for comparison of the largest and smallest litter mates as determined by birth weight and by weight at 5 weeks*

	Largest *v.* smallest		
	No. of muscle fibres	No. of primaries	S : P ratio
(*a*) by birth weight	$P > 0{\cdot}05$	$P > 0{\cdot}05$	$P < 0{\cdot}05$
(*b*) by 5-week weight	$P < 0{\cdot}05$	$P > 0{\cdot}05$	$P < 0{\cdot}01$

based on a comparison of extreme pairs of animals selected by birth weight. Comparison of large and small littermates, by birth weight, from each of the five litters used in this study was therefore carried out (Table 1). Although the S : P ratio was significantly reduced in the smallest littermates there was no significant difference between pairs for total muscle fibre number or primary fibre number. This was due to the fact that only two of the five litters produced animals which could be defined as runts by birth weight (less than 950 g). Handel and Stickland (1987) found significant fibre number differences between large and runt littermates, but not between large and small or between small and runt categories as defined by birth weight.

Of the five litters investigated here, two litters produced no birth weight runts and all animals grew at a comparable rate; one litter produced no birth weight runts but one animal grew very slowly and, at 5 weeks, was only proportionately 0·25 the weight of the largest littermate; and two litters produced animals defined as runts by virtue of their weight at birth. Relative growth rate seems to be more indicative of muscle fibre number differences since small birth weight pigs are able to reach similar weights to their larger littermates (at birth) if they have a relatively high fibre number (Handel and Stickland, 1988). Therefore, large and small littermates were also selected on the basis of their weight at 5 weeks (Table 1). By 5 weeks fibre number was significantly reduced ($P < 0{\cdot}05$) in the smaller littermates by a mean proportional difference of 0·30. Since there was no significant difference in primary fibre number this reduction seemed to be

TABLE 2

Correlation of total fibre number with number of primaries, and with secondary-to-primary fibre number ratio (S : P ratio) for all data, and by litter

	No.	No. of fibres *v.* no. of primaries	No. of fibres *v.* S : P ratio
Litter 251	9	r = 0·40	r = 0·68*
Litter 269	12	r = 0·92***	r = 0·81**
Litter 250	11	r = 0·66*	r = 0·79**
Litter 274	8	r = 0·90**	r = 0·51
Litter 242	8	r = 0·96***	r = 0·82*
Average		r = 0·77 r^2 = 0·59	r = 0·72 r^2 = 0·52

solely due to the significant decrease in S : P ratio from 25·16 to 20·84 ($P < 0·01$).

The correlation of total fibre number with number of primaries and with S : P ratio was investigated for each litter (Table 2). The average correlation coefficients were also calculated. The correlation coefficient obtained by combining all the data (Figure 3) was not considered to be a good indicator of the true variation within litters since this did not take into account the significant variations in primary fibre number between litters. The square of the correlation coefficient (r^2) was also calculated. This is an indication of the proportion of the variation in the dependent variable (muscle fibre number) which can be explained by the variation in the dependent variables (primary fibre number and S : P ratio). Values for r^2 of 0·59 and 0·52 respectively were obtained. Since all the variation in muscle fibre number must have been due to variation in either primary fibre number or secondary fibre number this suggested that both primary fibre number and secondary fibre number made an approximately equal contribution to total muscle fibre number variations within litters.

DISCUSSION

The results clearly demonstrated the large variation in myofibre number present in five litters of pigs (Figure 1). The importance of muscle fibre number as a determinant of muscle mass and post-natal growth is also emphasized (Figure 2) and supports earlier work (Miller *et al.*, 1975). The factors which cause this wide variation in fibre number are, therefore, of prime importance in the selection and rearing of meat animals.

Inter-litter variations in mean number of muscle fibres appeared to be largely attributable to the variation in primary fibre number (Figure 4) since variations in mean

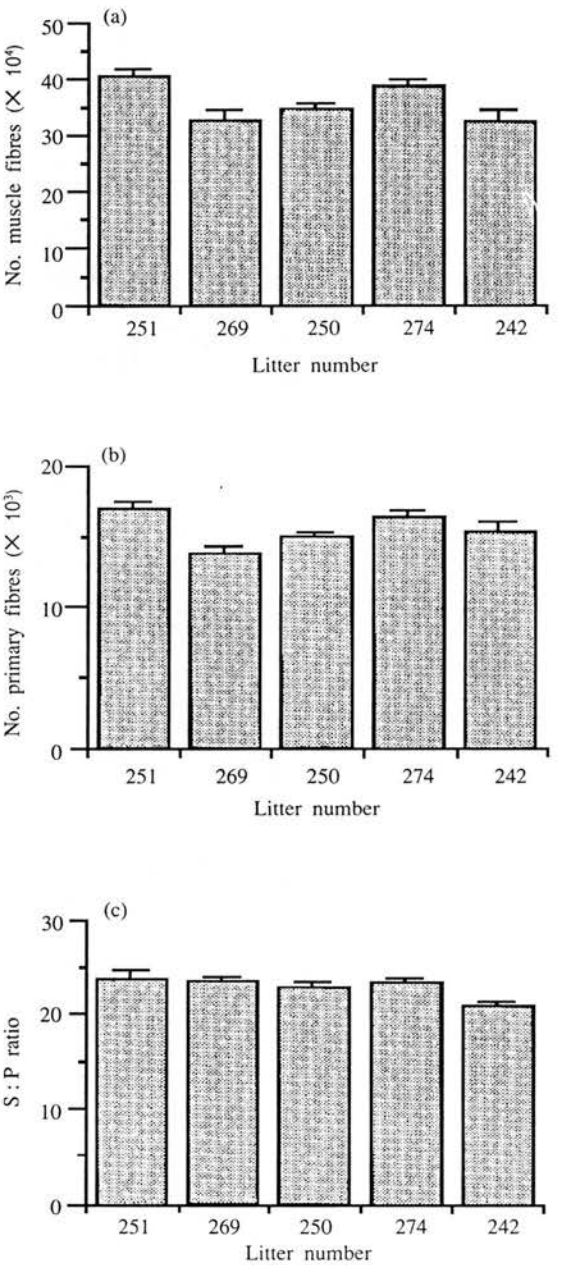

FIG. 4. Bar graphs of average muscle data for each litter (mean ± s.e.): (a) total number of muscle fibres ($P < 0·05$); (b) total number of primary fibres ($P < 0·01$); (c) secondary-to-primary fibre number ratio ($P > 0·05$).

S : P ratio, between litters, were not significant. This is consistent with the general belief that primary fibre number is a fixed genetic component of total muscle fibre number and its development is unaffected by conditions occurring *in utero* e.g. either nutritional deprivation (Handel and Stickland, 1987), or innervation (McLennan, 1983). Genetic variations in primary fibre number are known to occur between different breeds of pigs. Stickland and Handel (1986) compared Large White pigs with genetically small miniature pigs and found that any size difference was caused by a lower fibre number, due to a decreased primary fibre number and a lower S : P ratio. The contribution of a lowered primary fibre number was found to be four times more significant than S : P ratio in causing a reduced total fibre number. The results presented here suggest that significant within-breed variations in primary fibre number also exist although these are considerably smaller than those that exist between breeds.

The causes of intra-litter variation in muscle fibre number appear to be more complex with variations in both S : P ratio and primary fibre number playing a rôle. Comparison of large and small littermates, selected by birth weight, suggest that this difference is due to a lower S : P ratio in the smaller littermate (Handel and Stickland, 1987) in cases where low birth weight is associated with a reduced fibre number. Comparison was also made of animals selected at 5 weeks of age (Table 1). By this age some animals were seen to grow well whilst others only grew very slowly. This seemed to give some indication of their fibre number since low birth weight animals are able to exhibit catch-up growth to their larger littermates if they possess a relatively high number of muscle fibres (Handel and Stickland, 1988). When extreme pairs of animals were compared, using 5-week weight as a selection parameter, both fibre number and S : P ratio were significantly reduced. There was no significant variation in primary fibre number between littermate extremes selected by either birth weight or 5-week weight. Handel and Stickland (1987) noted that two severely runted animals in their study had a significantly reduced primary fibre number and therefore excluded them on the basis that they constituted a subpopulation of animals (Royston, Flecknell and Wootton, 1982). It is possible that these animals experience the combined disadvantage of a genetically lowered primary fibre number and pre-natal undernutrition which accounts for their extremely small size and low fibre number or that the undernutrition was so severe that it affected the primary fibre population.

Correlation of both primary fibre number and S : P ratio with total fibre number for all animals within each litter demonstrated that both varied significantly with fibre number. The square of the coefficients of correlation, calculated for primary fibre number and for S : P ratio, suggested that the variation in total muscle fibre number within litters may be due an approximately equal contribution from S : P ratio and primary fibre number. However, the variation between litters is quite high. This may be due to additional factors such as litter size and maternal metabolism which may affect foetal nutrition and therefore affect the relative importance of S : P ratio compared with primary fibre number in determining total muscle fibre number.

The underlying cause of small size in low birth-weight pigs is thought to be undernutrition *in utero*. This can be seen by the U-shaped distribution of foetal weights in each uterine horn which is believed to be a consequence of differing nutrition (McLaren and Mitchie, 1960; Perry and Rowell, 1969). Runt animals also have a lower placental weight and a reduced placental blood flow when compared with their larger littermates (Wootton, McFadyen and Cooper, 1977) indicating a reduced nutrient supply. Undernutrition *in utero* causes a reduction in fibre number by affecting the secondary fibre population (Wigmore and Stickland, 1983; Handel and Stickland, 1987). The effect of pre-natal undernutrition on fibre number is also well established under experimental conditions due to maternal food restriction (rats: Wilson, Ross and Harris, 1988; guinea-pig: Ward and Stickland, 1991) and these conclusions are supported by the results presented here (Table 1).

However, primary fibre number also seems

to vary within the litter (Table 2) to some extent although this variation does not appear to be influenced by nutrition since it has been demonstrated that there is no significant difference in primary fibre number between littermate extremes of birth weight, caused by nutritional differences *in utero* (Handel and Stickland, 1987) except possibly in cases of extreme runting. Small variations in primary fibre number tend to cause a proportionately greater effect than S : P ratio variations since they act as a template on which the secondary fibres form (Ashmore, Robinson, Rattray and Doerr, 1972; Harris, Duxson, Fitzsimmons and Rieger, 1989). Therefore, piglets in the mid range of birth weights may have similar S : P ratios, due to similar locations in the uterine horns and similar nutritional environments during gestation, but varying muscle fibre numbers due to small variations in primary fibre number caused by factors other than nutrition.

As a whole, when considered with the results of Handel and Stickland (1987), these results suggest that primary fibre number is a relatively more fixed genetic component than secondary fibre number, and is therefore more indicative of the genotype of an animal with respect to muscle. Secondary fibre number, although having a genetic component, is more vulnerable to environmental factors *in utero*. Both primary fibre and secondary fibre variations seem to make an equal contribution to the muscle phenotype.

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REFERENCES

ASHMORE, C. R., ADDIS, P. B. and DOERR, L. 1973. Development of muscle fibres in the fetal pig. *Journal of Animal Science* **36:** 1088-1093.

ASHMORE, C. R., ROBINSON, D. W., RATTRAY, P. V. and DOERR, L. 1972. Biphasic development of muscle fibers in the fetal lamb. *Experimental Neurology* **37:** 241-255.

DWYER, C. M. and STICKLAND, N. C. 1989. The determinants of inter- and intra-litter variations in muscle fibre number in the pig. *Journal of Anatomy* **167:** 264 (Abstr.).

GOLDSPINK, G. and WARD, P. 1979. Changes in rodent fibre types during postnatal growth, undernutrition and exercise. *Journal of Physiology* **296:** 453-469.

GUTH, L. and SAMAHA, F. J. 1970. Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28:** 365-367.

HANDEL, S. E. and STICKLAND, N. C. 1987. Muscle cellularity and birth weight. *Animal Production* **44:** 311-317.

HANDEL, S. E. and STICKLAND, N. C. 1988. Catch-up growth in pigs: a relationship with muscle cellularity. *Animal Production* **47:** 291-295.

HARRIS, A. J., DUXSON, M. J., FITZSIMMONS, R. B. and RIEGER, F. 1989. Myonuclear birthdates distinguish the origins of primary and secondary myotubes in embryonic mammalian skeletal muscle. *Development* **107:** 771-784.

HEGARTY, P. V. J. and ALLEN, C. E. 1978. Effect of pre-natal runting on the post-natal development of skeletal muscles in swine and rats. *Journal of Animal Science* **46:** 1634-1640.

LUFF, A. R. and GOLDSPINK, G. 1970. Total number of fibers in the muscles of several strains of mice. *Journal of Animal Science* **30:** 891-893.

MCLAREN, A. and MITCHIE, D. 1960. Control of pre-natal growth in mammals. *Nature, London* **187:** 363-365.

MCLENNAN, I. S. 1983. Neural dependence and independence of myotube production in chick hindlimb muscles. *Developmental Biology* **98:** 287-294.

MILLER, L. R., GARWOOD, V. A. and JUDGE, M. D. 1975. Factors affecting porcine muscle fiber type, diameter and number *Journal of Animal Science* **41:** 66-77.

PERRY, J. S. and ROWELL, J. G. 1969. Variations in foetal weight and vascular supply along the uterine horn of the pig. *Journal of Reproduction and Fertility* **19:** 527-534.

ROYSTON, J. P., FLECKNELL, P. A. and WOOTTON, R. 1982. New evidence that the intrauterine growth retarded piglet is a member of a discrete subpopulation. *Biology of the Neonate* **42:** 100-104.

STAUN, H. 1963. Various factors affecting number and size of muscle fibres in the pig. *Acta Agriculturae Scandinavica* **13:** 293-322.

STICKLAND, N. C. and GOLDSPINK, G. 1973. A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. *Animal Production* **16:** 135-146.

STICKLAND, N. C. and HANDEL, S. E. 1986. the numbers and types of muscle fibres in large and small breeds of pigs. *Journal of Anatomy* **147:** 181-189.

STICKLAND, N. C., WIDDOWSON, E. M. and GOLDSPINK, G. 1975. Effects of severe energy and protein deficiencies on the fibres and nuclei in skeletal muscles of pigs. *British Journal of Nutrition* **34:** 421-428.

SWATLAND, H. J. 1973. Muscle growth in the fetal and neonatal pig. *Journal of Animal Science* **37:** 536-545.

WARD, S. S. and STICKLAND, N. C. 1991. Why are slow and fast muscles differentially affected during prenatal undernutrition? *Muscle and Nerve* In press.

WIGMORE, P. M. C. and STICKLAND, N. C. 1983. Muscle development in large and small pig fetuses. *Journal of Anatomy* **137:** 235-245.

WILSON, S. J., ROSS, J. J. and HARRIS, A. J. 1988. A critical period for the formation of secondary myotubes defined by prenatal undernourishment in rats. *Development* **102:** 815-821.

WOOTTON, R., MCFADYEN, I. R. and COOPER, J. E. 1977. Measurement of placental bloodflow in the pig and its relation to placental and foetal weight. *Biology of the Neonate* **31:** 333-339.

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Satellite cell content in muscles of large and small mice

S. C. BROWN AND N. C. STICKLAND

Department of Veterinary Basic Sciences, Royal Veterinary College, London, UK

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ABSTRACT

Quantitative analyses of the satellite cell content in the biceps brachii muscle of mice genetically selected for high (QL) and low (QS) bodyweight showed that selection alters the total number of satellite cell nuclei rather than the relative proportions of nuclei in the myofibre and satellite cell populations. These findings are in accordance with those previously published for other tissues of these mice and support the hypothesis that regulatory mechanisms remain unaltered by selection pressure. Size at birth, however, is a reflection of nutritional status as well as genetic background, and comparisons between differently sized littermates within each of the lines showed a significant increase in satellite cell density in larger compared with smaller individuals. These differences between littermates were not accompanied by any alteration in myofibre nuclear density. It is therefore suggested that whilst both genetic and nutritional factors exert their effects on muscle growth through an influence on satellite cell division, both do so at different stages during the programme of satellite cell differentiation.

INTRODUCTION

The role of nuclear proliferation during the postnatal growth of muscle was originally recognised through the work of Enesco & Puddy (1964) who showed that fibre hypertrophy was accompanied by an increase in the total number of nuclei. Extensive autoradiographic studies carried out both in vivo and in vitro have since identified the satellite cell as the sole source of nuclei added to the muscle fibre postnatally (Moss & Leblond, 1971; Bischoff, 1975, 1986*a*, *b*). Since between 50% and 99% of the total nuclear content of the muscle accumulates postnatally (Allen et al. 1979), and there is evidence to suggest that DNA accretion precedes protein accretion (Moss et al. 1968*a*, *b*), it seems that the satellite cell population represents some considerable potential for the postnatal growth of skeletal muscle.

Satellite cells characteristically lie wedged between the sarcolemma and basal lamina of the muscle fibre (Mauro, 1961). In young fast growing animals these cells display a high rate of division and may contribute up to 32% of the total myogenic nuclear population (Schultz, 1974; Cardasis & Cooper, 1975). However, during the early stages of postnatal growth, more than 50% of the cells produced by satellite cell division become incorporated into the muscle fibre, thereby successively reducing the satellite cell percentage to around the 4% level seen in the adult (Schultz, 1972, 1974; Ontell, 1974; Cardasis & Cooper, 1975). Whereas the quantitative differences associated with growth are well documented, the contribution made by this cell population to genetically determined differences in muscle mass has remained unclear.

The present study therefore set out to compare the satellite cell population in the biceps brachii muscle of Q strain mice genetically selected for high (QL) and low (QS) bodyweight (Falconer, 1973). These mice diverge in size around the time of birth (Rucklidge, 1981), after which their markedly different growth rates result in animals differing by as much as 63% in adult bodyweight. This pattern of growth suggests that comparison of high and low birthweight individuals both within and between each of the lines provide a situation for determining whether actual body size or genetic potential is more important in determining the satellite cell content of a given muscle. Biceps brachii was selected for such a study since fibre

Correspondence to Dr S. C. Brown, Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK.

Table 1. *Transverse sectional area of muscle fibres in biceps brachii of newborn QL and QS mice*

Line	Littermate	Mean transverse sectional area*
QL	Large	71.67±5.06
QL	Small	73.06±11.6
QS	Large	79.16±4.31
QS	Small	84.24±11.8

* Figures represent the mean(±S.E.M.) of measurements taken from 4 individuals.

formation is complete by the time of birth, thereby avoiding any confusion between myoblasts, which will form new fibres, and satellite cells.

MATERIALS AND METHODS

The largest and smallest littermate, on the basis of weight, were selected at birth from each of 5 litters of high bodyweight (QL) and 5 litters of low bodyweight (QS). Animals were killed by decapitation and all limbs initially fixed in the resting position for 15 min in 0.1 M sodium cacodylate buffer, pH 7.3, containing 2% glutaraldehyde. The muscle was then dissected from the limb, fixed for a further 3 h in 2% glutaraldehyde, washed in cacodylate buffer for 1 h, fixed for 1.5 h in an aqueous solution containing 0.6% osmium tetroxide and 0.4% potassium ferrocyanide (Aguas, 1982), washed in distilled water, dehydrated through graded acetones up to 100%, infiltrated and embedded in Araldite resin (CY212) which was polymerised at 60 °C for 4–6 d.

Before comparisons could be made regarding satellite cell content, measurements of fibre transverse sectional area and nuclear lengths had to be made since any differences in these parameters would affect the estimations of satellite cell content.

Semithin (1 μm) sections were taken from the midbelly of the muscle and stained with 1% methylene blue. Measurements of transverse sectional area were made on 150 fibres selected across the girth of each sectioned muscle using a Kontron MOP Videoplan interactive image analysis system. Measurements of nuclear length in 1 littermate from each of the QL and QS lines were carried out on 200 nuclei sectioned in 1 μm longitudinal sections. Ultrathin transverse sections (90 nm) were taken from the midbelly portion of each muscle and collected on 100 mesh copper grids. Sections were stained in 1% uranyl acetate (40 min) followed by Reynolds (1963) lead citrate (5 min), and examined using a JEOL 1200X electron microscope operating at an accelerating voltage of 80 kV. Randomly selected areas of muscle were chosen and 200 fibres evaluated for the presence of myofibre or satellite cell nuclei. The criteria used for the identification of satellite cells were those employed by Ontell (1977), namely, the presence of a heterochromatic nucleus compared with that of the myofibre, enclosure within the basal lamina of the fibre, close apposition to the surface of the fibre and a complete absence of myofibrils (Fig. 1).

Fig. 1. Transverse section of a myofibre and associated satellite cell (S) in the newborn biceps brachii muscle.

RESULTS

Morphological description of satellite cells in the newborn QL and QS

Figure 1 shows a single mature myofibre with an attendant satellite cell typically found in the newborn mouse biceps brachii. The euchromatic myofibre nucleus, which is peripherally located within the fibre, contrasts with the more heterochromatic satellite cell nucleus. Both satellite cell and myofibre are enclosed within a common basal lamina, none of which penetrates between the closely opposed membranes of each cell. Also clearly identifiable is an immature myotube closely apposed to an adjacent myofibre. This myotube appears to be similar to the satellite myotubes previously described by Ontell & Dunn

Table 2. *Satellite cell content of biceps brachii in newborn QL and QS*

Line	Relative size of litter-mates	n	Body-weight	Incidence of myofibre nuclei*	Number of satellite cell nuclei per 200 fibres	% Number of nuclei within the satellite cell population
QL	Large	5	1.8±0.07	0.36±0.02	25.60±2.3	26.2±1.3
	Small	5	1.4±0.1	0.31±0.03	17.46±1.8	22.4±2.5
QS	Large	5	1.42±0.03	0.35±0.03	21.98±2.76	23.6±2.8
	Small	5	1.26±0.04	0.33±0.02	12.38±1.48	16.2±1.7

* Population of myofibres containing a muscle nucleus in section.

(1978) in newborn rat muscle. The centrally located nucleus is indistinguishable from that of the adjacent myofibre, and myofibrils are scattered throughout the cytoplasm. The larger gap between this myotube and its parent myofibre, accentuated in this case by a cellular process, acts as a further feature which distinguishes the satellite myotube from the satellite cell.

Measurements of fibre transverse sectional area

A 2-way analysis of variance showed that fibre transverse sectional area was not significantly different between either the QL or QS or the large and small littermates. The means±S.E.M. are shown in Table 1.

Nuclear length measurements

Nuclear length in longitudinal section was not significantly different between the 2 lines as determined by a U test of the length distributions of 200 randomly selected nuclei (the mean values for the QL and QS were 10.24±0.19 and 9.824±0.21 μm respectively).

Comparisons of satellite content in the 2 lines could therefore be made assuming the absence of any bias due to differences in fibre transverse sectional area or nuclear length.

Table 2 summarises the nuclear content of biceps brachii for 5 large and 5 small individuals taken from each of the QL and QS lines. The incidence of myofibre nuclei (proportion of fibres displaying a fibre nucleus in transverse section) was not significantly different either between the QL and QS or between the large and small littermates (see Table 3).

The percentage of satellite cell nuclei relative to the number of myonuclei was not significantly different between the QL and QS, although there was a significant difference between large and small littermates (Tables 2, 3). Since there were no significant differences in the myonuclear population either between the lines or littermates this follows the same trend as the number of satellite cell nuclei per 200 fibres.

Table 3. *Results of 2-way analysis of variance to test for the effect of line (QL/QS) and littermate size (large/small) on nuclear content in biceps brachii in the newborn*

	QL/QS	Large/small
Incidence of myofibre nuclei	ns	ns
Number of satellite nuclei per 200 fibres	ns	Significant (1%)
% Number of nuclei within the satellite cell population	ns	Significant (2.5%)

ns, nonsignificant.

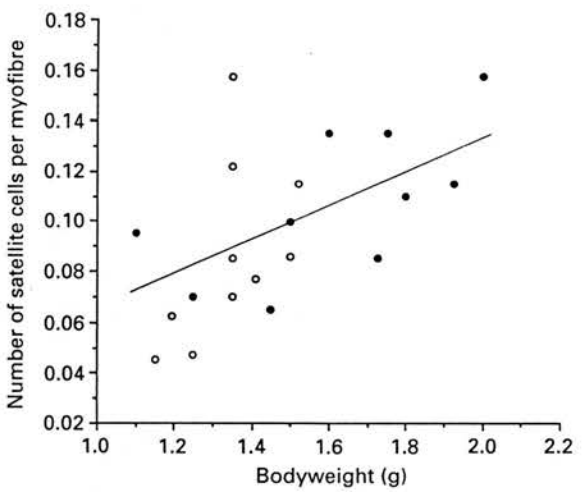

Fig. 2. Relationship between the number of satellite cells per myofibre and body weight in newborn QL (●) and QS (○) mice. The line is the calculated regression line for the relationship in the QL mice.

The number of satellite cell nuclei observed in a transverse section of 200 fibres was converted to the number of satellite cells per myofibre and presented graphically in Figure 2. The regression equations which describe the relationship between the number

Table 4. *Percentage number of satellite cell nuclei associated with myofibre nuclei*

Line	Relative size	% of satellite cell nuclei*
QL	Large	27.2±5.08
QL	Small	24.4±4.18
QS	Large	27.6±4.4
QS	Small	17.6±4.12

* Based on counts of 200 fibres.

Table 5. *Contingency table to test for the preferential association of satellite with myofibre nuclei*

	No. of myofibres without a myofibre nucleus	No. of myofibre with a myofibre nucleus	Total
No. of fibres with a satellite cell nucleus	266 [300]	95 [61]	361
No. of fibres without a satellite cell nucleus	2730 [2696]	509 [543]	3239
Total	2996	604	3600

Expected values assuming no association between the 2 populations of nuclei are shown in square brackets.

of satellite cells per myofibre (N) and birthweight (BW) were as follows:

QL: $N = -0.0087 \pm 0.0714\ BW$ Rsq 44.9%,
QS: $N = 1.17 \pm 2.02\ BW$ Rsq 34.6%.

A t test of the regression coefficients showed that these 2 parameters were significantly associated within the QL ($P = 0.034$) but not the QS ($P = 0.074$). Accordingly only the QL regression line is shown in Figure 2.

Association of satellite nuclei with myofibre nuclei

Table 4 shows the mean percentage number of satellite nuclei which were associated in the same section with a myofibre nucleus in 5 large and small littermates taken from each of the QL and QS lines. Analysis of these data (1-way ANOVA after angular transformation) showed there to be no significant difference between the number of satellite nuclei associated with myofibre nuclei in either of these groups. It was therefore considered appropriate to combine the data to test whether there was a preferential association between satellite and myofibre nuclei irrespective of animal group (Table 5). A χ^2 analysis carried out on the data was highly significant ($P < 0.001$), providing strong evidence of a preferential association between satellite and myofibre nuclei.

DISCUSSION

Comparisons between the QL and QS

During the early stages of postnatal growth the density of satellite cells may either increase or decrease, depending on the growth pattern of the muscle (Gibson & Schultz, 1983). This has led to the suggestion that each satellite cell division does not regularly result in one daughter cell fusing with the fibre and the other continuing as a satellite cell (Campion, 1984). Interestingly, selection for divergent body size seems not to have altered this pattern of fusion, since the proportion of muscle nuclei belonging to the satellite cell population was the same in both the QL and QS. Indeed, previous work in the high and control bodyweight lines of the Japanese quail found that during growth the number of satellite cells increased in proportion with the length of the muscle fibres, thereby effecting no change in the percentage of satellite nuclei between the lines at any age (Campion et al. 1982*b*). Nonetheless, the total nuclear content of the biceps brachii at birth in these mice does differ as a consequence of the divergence which they show in fibre number (Penney et al. 1983). This situation has been shown in the Japanese quail (*Coturnix coturnix japonica*) where selection for high body weight results in an increase in fibre number and length (Fowler et al. 1980) but not nuclear density which is unchanged (Campion et al. 1982*b*). It therefore seems that in both mice and quails a genetically determined increase in muscle weight is accomplished through an increase in the number of satellite cell segments (a single satellite cell plus the number of nuclei contained in the adjacent segment of fibre) rather than through any alteration in the satellite cell segment size.

A number of authors have noted an association between the nuclei of the myofibre and those of the attendant satellite cell (Ontell, 1974; Schmalbruch & Hellhammer, 1977). These muscle and satellite cell nuclei 'doublets' were suggested by Ontell (1974) to come about as a result of one daughter cell of a satellite cell division fusing with the fibre, and the other remaining within the satellite cell population. In the present study between 23% and 28% of the satellite nuclei were found to be associated with the nuclei of the principal fibre, a proportion which did not differ significantly between the QL or QS. Indeed, if the hypothesis of Ontell (1974) is correct, no

difference between the QL and QS would be expected, since the proportion of satellite cells which are incorporated into the muscle fibre after each round of division appears not to be altered by selection.

Cell proliferation plays an important role in muscle growth both prenatally, when the number of myoblasts available for fusion may determine the number of fibres that form (Penney et al 1983), and postnatally when DNA accretion becomes a fundamental process in fibre hypertrophy (Allen et al. 1979; Campion et al. 1982*b*; Purchas et al. 1985). The evidence presented here suggests that differences in fibre number and length between the QL and QS at birth must be the consequence of alterations in the rate of cell proliferation prenatally. However, confirmation of this awaits a study of prenatal development in these mice.

Comparisons between large and small littermates

Satellite cell density tended to be correlated with body weight although this was only significant in the QL line, with the largest (heaviest) littermate containing the highest density of satellite cell nuclei. Since both the QL and QS were inbred, thereby minimising genetic heterogeneity between littermates, a large proportion of this variation may be attributed to the intrauterine environment (McLaren, 1965). Such intrauterine effects are due to the variation in the blood supply at different positions within the uterine horn (McLaren & Mitchie, 1960), and appear to be manifested principally during the latter half of gestation when the nutritional demands of the fetus are greatest (Widdowson, 1980). The smallest littermate within an inbred line of mice may therefore be assumed to be small as a result of an inadequate plane of nutrition. It is consequently interesting to note that the effects of undernutrition appeared to be manifested principally in a reduction in the density of satellite cells, whilst the density of myonuclei remained unchanged. This latter observation might have been expected in view of the close parallel which has been shown to exist between DNA accretion and protein synthesis during muscle growth (Moss, 1968*a*; Allen et al. 1979). However, differences in fibre length (and therefore the total number of myonuclei) were evident between divergently sized littermates (personal observation), which implied that an alteration in the density of satellite cells had affected the number of nuclei able to fuse with the growing fibre. Whilst a reduction in DNA accretion as a response to an adverse nutritional environment has been noted by a number of other investigators (Moss, 1968*b*; Cheek et al. 1971; Campion et al. 1982*a*), a direct relationship between this response and the number of satellite cells has not previously been demonstrated.

The embryological origin of the satellite cell population remains unclear, although Cossu et al. (1988) claim to have identified this cell type at 16 d gestation in the mouse. Whilst such results require further confirmation, it is interesting to note that the appearance of this cell population during the later stages of gestation coincides with that period of fetal life which is most associated with nutritional constraint. It might therefore be envisaged that the reduced nuclear content of the smallest littermate came about as a direct consequence of a gradual reduction in the rate of satellite cell division during the later stages of gestation, a reduction which in itself was determined by the level of nutrition of each fetus.

Conclusions

The muscle mass attained by any given individual is dependent on both genetic and nutritional factors, both of which appear to operate through an effect on the availability of nuclei. Other factors such as mechanical activity, which may act postnatally, are not considered in this investigation. Quantitative analyses of the satellite cell content of mice which display genetically determined differences in bodyweight indicate that selection fails to alter the relative proportions of nuclei in the myofibre and satellite cell compartments. In contrast comparisons between differently sized littermates show an increase in the density of satellite cell nuclei within the larger individuals. This appears to suggest that adverse nutritional circumstances may alter the rate of cell division within the satellite cell compartment, although as the proportion of myonuclei sectioned within the fibres seems to show, this exerts no effect on the density of nuclei within the fibre itself. These findings are in general agreement with the hypothesis proposed by Falconer et al. (1978) which suggests that whilst selection for divergent bodyweight may act upon component cellular processes, such as the rate of cell division, the regulatory mechanisms, i.e. those which determine such factors as the proportion of nuclei which fuse with the fibre, remain unchanged.

REFERENCES

AGUAS AP (1982) The use of osmium tetroxide-potassium ferrocyanide as an extracellular tracer in electron microscopy. *Stain Technology* **57**, 69–73.

ALLEN RE, MERKEL RA, YOUNG RB (1979) Cellular aspects of muscle growth: myogenic cell proliferation. *Journal of Animal Science* **49**, 115–127.

BISCHOFF R (1975) Regeneration of single skeletal muscle fibres *in vitro*. *Anatomical Record* **182**, 215–236.

BISCHOFF R (1986*a*) Proliferation of muscle satellite cells on intact myofibers in culture. *Developmental Biology* **115**, 129–139.

BISCHOFF R (1986*b*) A satellite cell mitogen from crushed adult muscle. *Developmental Biology* **115**, 140–147.

CAMPION DR (1984) The muscle satellite cell: a review. *International Review of Cytology* **87**, 225–251.

CAMPION DR, MARKS HL, REAGAN JO, BARRETT JB (1982*a*) Composition and muscle cellularity of Japanese Quail after selection for high body weight under an optimal or suboptimal nutritional environment. *Poultry Science* **61**, 212–217.

CAMPION DR, MARKS HL, RICHARDSON LR (1982*b*) An analysis of satellite cell content in the semimembranosus muscle of Japanese quail (*Coturnix coturnix japonica*) selected for rapid growth. *Acta Anatomica* **112**, 9–13.

CADASIS CA, COOPER GW (1975) An analysis of nuclear numbers in individual muscle fibres during differentiation and growth: a satellite cell-muscle fibre growth unit. *Journal of Experimental Zoology* **191**, 347–358.

CHEEK DB, HOLD AB, HILL DE, TALBERT JL (1971) Skeletal muscle cell mass and growth – the concept of the deoxyribonuclease acid unit. *Paediatric Research* **5**, 312–328.

COSSU G, RANALDI G, SENNI MJ, MOLVIARO M, VIVARELLI E (1988) 'Early' mammalian myoblasts are resistant to phorbol ester induced block of differentiation. *Development* **102**, 65–69.

ENESCO M, PUDDY D (1964) Increase in the number of nuclei and weight in skeletal muscle of rats of various ages. *American Journal of Anatomy* **114**, 235–244.

FALCONER DS (1973) Replicated selection for body weight in mice. *Genetic Research*, Camb. 22. pp 291–321.

FALCONER DS, GAULD IK, ROBERTS RC (1978) Cell numbers and cell sizes in organ of mice selected for large and small body size. *Genetic Research (Cambridge)* **31**, 287–301.

FOWLER SP, CAMPION DR, MARKS HL, REAGAN JO (1980) An analysis of skeletal muscle response to selection for rapid growth in Japanese Quail (*Coturnix coturnix Japonica*). *Growth* **44**, 235–252.

GIBSON MC, SCHULTZ E (1983) Age related differences in absolute numbers of skeletal muscle satellite cells. *Muscle and Nerve* **6**, 574–580.

MAURO AL (1961) Satellite cell of skeletal muscle fibres. *Journal of Biophysical and Biochemical Cytology* **9**, 493–495.

MCLAREN A (1965) Genetic and environmental effects on foetal and placental growth in mice. *Journal of Reproduction and Fertility* **9**, 79–98.

MCLAREN A, MITCHIE D (1960) Control of prenatal growth in mammals. *Nature* **187**, (4735) 363–365.

MOSS FP (1968*a*) The relationship between the dimensions of the fibres and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. *American Journal of Anatomy* **122**, 535–564.

MOSS FP (1968*b*) The relationship between the dimensions of the fibres and the number of nuclei during restricted growth, degrowth and compensatory growth of skeletal muscle. *American Journal of Anatomy* **122**, 565.

MOSS FP, LEBLOND CP (1971) Satellite cells as the source of nuclei in muscles of growing rats. *Anatomical Record* **170**, 421–436.

ONTELL M (1974) Muscle satellite cells: a validated technique for light microscopic identification and a quantitative study of changes in their population following denervation. *Anatomical Record* **178**, 211–228.

ONTELL M (1977) Neonatal muscle: an electron microscopy study. *Anatomical Record* **178**, 211–228.

ONTELL M, DUNN RF (1978) Neonatal muscle growth: a quantitative study. *American Journal of Anatomy* **152**, 539–556.

PENNEY RK, PRENTIS PF, MARSHALL PA, GOLDSPINK G (1983) Differentiation of muscle and the determination of ultimate tissue size. *Cell and Tissue Research* **228**, 375–388.

PURCHAS RW, ROMSOS DR, ALLEN RE, MERKEL RA (1985) Muscle growth and satellite cell proliferative activity in obese (ob/ob) mice. *Journal of Animal Science* **60**, 644–651.

REYNOLDS ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

RUCKLIDGE GJ (1981) Differences in body compositions, growth and food intakes between mice which have been selected for a small and large body size. *British Journal of Nutrition* **46**, 441–450.

SCHMALBRUCH H, HELLHAMMER U (1977) The number of nuclei in adult rat muscles with special reference to satellite cells. *Anatomical Record* **189**, 169–176.

SCHULTZ E (1972) Changing satellite cell populations in skeletal muscle of the neonatal mouse. *Anatomical Record* **172**, 401.

SCHULTZ E (1974) A quantitative study of the satellite cell population in postnatal mouse lumbrical muscle. *Anatomical Record* **180**, 589–596.

WIDDOWSON EM (1980) Definitions of growth. In *Growth in Animals – Studies in the Agricultural and Food Sciences* (ed. T. L. J. Lawrence). London: Butterworths.

Muscle Cellularity and Postnatal Growth in the Pig[1]

Catherine M. Dwyer*, [2], John M. Fletcher†, and Neil C. Stickland*

*Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 OTU and
†Unilever Research, Colworth House, Sharnbrook, Bedfordshire MK44 1LQ, U.K.

ABSTRACT: Fast-growing strains of pigs and of other animals tend to have more muscle fibers than do slow-growing strains. The relationship between fiber number and growth rate was extended by examining pigs within the same strain. Seven litters of pigs (n = 66) were weighed at monthly intervals from birth to approximately 80 kg. The semitendinosus muscle was removed and an estimation of total fiber number, total primary fiber number, and mean secondary:primary fiber number (S:P) ratio was made for each animal. Pig growth was divided into three approximately linear phases: birth to 6 kg, 6 to 25 kg, and 25 kg to slaughter weight. Average daily gain in the first two phases was correlated with birth weight ($r = .3614$, $P < .05$ and $r = .5873$, $P < .001$, respectively) but was not correlated with total muscle fiber number. In the third growth phase ADG was correlated with muscle fiber number ($r = .4149$, $P < .001$) and was not correlated with birth weight. The gain:feed ratio was also calculated for each pig during the third growth phase and was found to be correlated with fiber number ($r = .4191$, $P < .001$). These results suggest that muscle fiber number is an important determinant of postnatal growth such that pig littermates with a high fiber number tend to grow faster and more efficiently than littermates with a lower fiber number.

Key Words: Pigs, Myofibers, Weight Gain, Feed Conversion Efficiency

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Introduction

Muscle fiber number is known to be an important determinant of muscle mass (Luff and Goldspink, 1970; Miller et al., 1975). In addition, faster-growing strains of many animals tend to have more muscle fibers than do slower-growing strains (e.g., pig: Ezekwe and Martin, 1975; Miller et al., 1975). Comparison of Large White pigs with Göttingen mini-pigs suggests that the differences in muscle size are solely due to differences in myofiber number (Stickland and Handel, 1986); mini-pigs had a reduced primary fiber population and a reduced mean ratio of secondary fibers to primary fibers (**S:P ratio**). Between strains, therefore, it seems that myofiber number is important in determining muscle mass, mature size, and growth rate. There is some evidence to suggest that a similar relationship exists within a litter because runt pigs tend to grow more slowly and less efficiently than their larger littermates (Powell and Aberle, 1980). These animals have been shown to have a reduced muscle fiber number, caused by a reduced secondary fiber population, compared with their largest littermate (Wigmore and Stickland, 1983; Handel and Stickland, 1987).

The purpose of the present investigation was to extend these observations by examining the growth rate and muscle fiber numbers of all pigs within a number of litters drawn from the same herd. The hypothesis was that muscle fiber number determines the postnatal growth rate of animals and the efficiency of that growth within the same strain.

Materials and Methods

Seven litters of Large White × Landrace pigs were bred and reared under similar commercial conditions. Pigs were weighed at birth and at 1-mo intervals until they reached approximately 80 kg in weight. Pigs were weaned at 3 wk and housed thereafter in groups of three to four pigs of similar weight. Pigs were given ad libitum access to a commercial weaning diet (8% oil, 21.5% CP, 2% fiber, 15.4 MJ per kg of diet) until they reached 5 kg in weight. They were then fed a commercial growing diet (5.5% oil, 22% CP, 3% fiber,

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[2]To whom correspondence should be addressed.

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14.4 MJ per kg of diet) to 26 kg and a commercial finishing diet (4.5% oil, 20% CP, 3.5% fiber, 13.9 MJ per kg of diet) to slaughter weight. Male pigs remained intact. Pigs were housed in well-ventilated, straw-bedded pens at a density of one pig per square meter. Weaned pigs were kept at 20 to 23°C until they reached 15 kg in weight, and thereafter at 15 to 20°C. Pigs were killed at 80 kg by standard abattoir procedures at the University of Nottingham slaughterhouse. The left semitendinosus muscle was removed from each animal (n = 66) and trimmed of fat and connective tissues. A whole mid-belly slice, of approximately 5 mm, was rapidly frozen in dichlorodifluoromethane cooled to its freezing point of −158°C in liquid nitrogen. Sections of 10 μm thickness were cut at −25°C and stained for myosin adenosine triphosphatase activity, using a modification of the method of Guth and Samaha (1970).

For each animal the total cross-sectional area of the semitendinosus muscle was first measured. The number of primary muscle fibers and total number of myofibers were then counted in approximately 50 randomly selected microscope fields at low power. The number of fibers counted represented 3 to 5% of the total number present. These data were used to estimate the total number of fibers, the total number of primary fibers, and the mean ratio of secondary fibers:primary fibers in the whole muscle. A Seescan image analysis system was used throughout (Seescan plc, Cambridge, U.K.). The regression of pig weight on age was calculated. No significant differences in growth rate were found between pigs housed in groups of three or groups of four; therefore, pen effects were considered to be negligible. Simple correlations of total fiber number with birth weight and growth rate were calculated using an Oxstat statistics package (Microsoft). An individual pig was considered to be an experimental unit. Littermate extremes were compared using a Student's paired *t*-test.

Results

Mature pigs exhibit a unique histochemical arrangement of muscle fibers consisting of central groups of slow fibers surrounded by large numbers of fast fibers. From previous studies (Wigmore and Stickland, 1983) it is known that one of the central slow fibers in each cluster develops as a primary myofiber, whereas all others are secondary myofibers. This means, therefore, that the number of primary fibers corresponds to the number of clusters of slow fibers. Thus, the number of primary and secondary fibers that formed prenatally was determined in the postnatal animal.

Growth of pigs was divided into three main phases, during which growth was approximately linear (Figure 1). The first phase was from birth to 6 kg (weaning weight), the second from 6 to 25 kg (10 wk old), and the third from 25 kg to slaughter weight. These phases coincided with changes in diets as outlined in the Materials and Methods section. Furthermore, an age of 70 d (equivalent to a weight of 25 kg in this study) has been identified as the period when growth is determined by the pig's own genotype (Blunn et al., 1953). This is supported by a more recent study in which pigs subjected to different treatments differed in ADG only after 70 d of postnatal growth (Pond et al., 1985). For these reasons it was decided to divide the postweaning growth into two phases. Growth rate was approximately doubled in successive phases (regression slopes were .24 ± .011, $P < .001$ for Phase 1; .48 ± .060, $P < .001$ for Phase 2; and .89 ± .132, $P < .001$ for Phase 3). Slopes increased significantly between Phase 1 and Phase 2 ($P < .001$) and between Phase 2 and Phase 3 ($P < .005$).

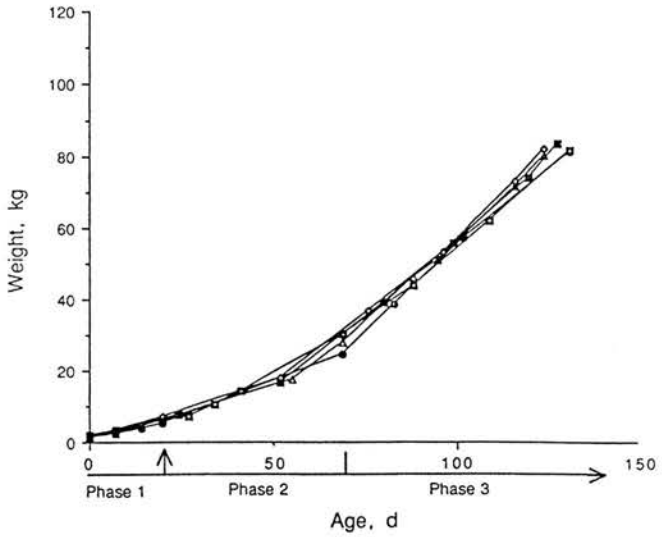

Figure 1. Live weight against age for all pigs (n = 66) from birth to approximately 80 kg. Values are litter means at each age. Growth was divided into three approximately linear phases: birth to 6 kg, 6 to 25 kg, and 25 kg to slaughter. Vertical arrow indicates weaning age (21 d).

Effects of Birth Weight. The influence of birth weight on ADG for each of the three growth phases is shown in Figure 2. Average daily gain during the first two growth phases was significantly correlated with birth weight (Figures 2a and b). Correlation coefficients of .361 ($P < .05$) for the first phase and .587 ($P < .001$) for the second phase were obtained. In the third growth phase there was no correlation of growth rate with birth weight (Figure 2c).

Effects of Myofiber Number. The correlation of total myofiber number with ADG is shown in Figure 3. There was no correlation between ADG and muscle fiber number for the first two phases (Figures 3a and b). However, in the third growth phase, fiber number was correlated with growth rate (r = .415, $P < .001$;

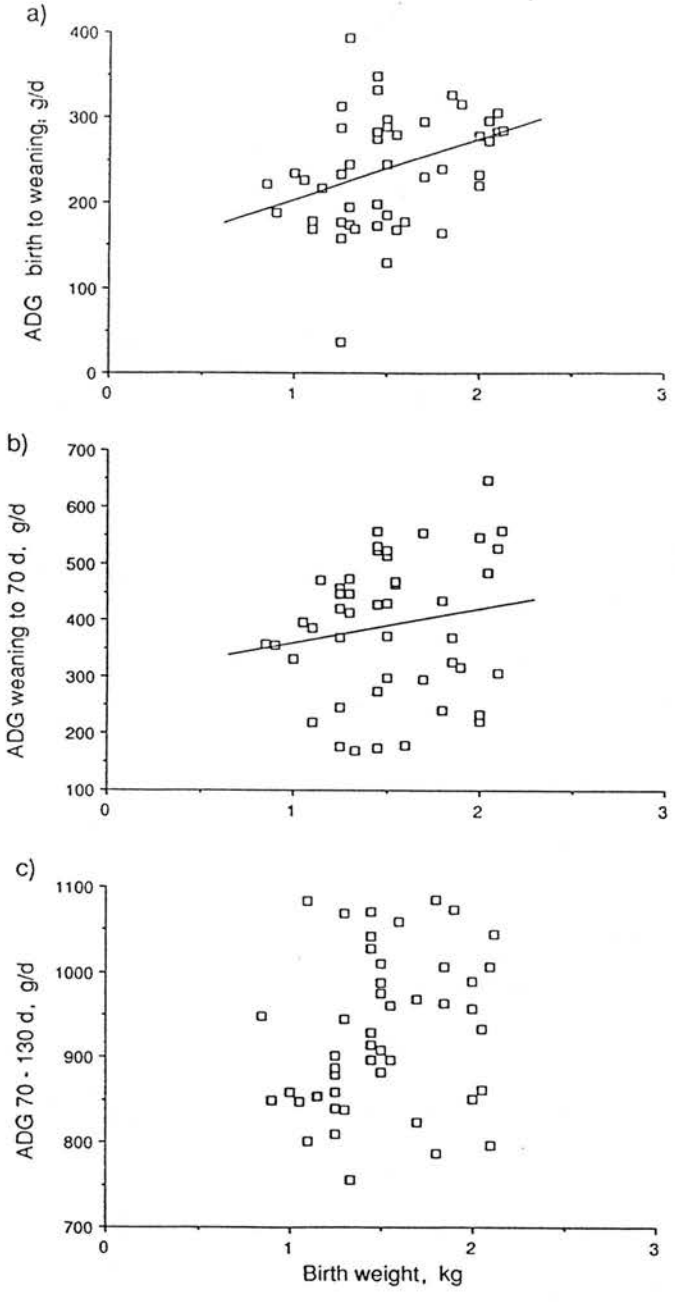

Figure 2. Average daily gain against birth weight (n = 47) for (a) Phase 1, birth to 6 kg; (b) Phase 2, 6 to 25 kg; and (c) Phase 3, 25 to 80 kg. Correlations were significant for Phase 1 (r = .361, $P < .05$) and Phase 2 (r = .587, $P < .001$).

Figure 3c). Although both primary fiber number and S:P ratio were significantly correlated with total muscle fiber number (r = .764 and r = .409 respectively, $P < .001$), only S:P ratio was directly correlated with ADG in the third phase (r = .439, $P < .001$). The gain:feed ratio was calculated for all pigs during the growth phase from 25 kg to slaughter weight. This was found to be correlated with total muscle fiber number (r = .419, $P < .001$; Figure 4). There was no correlation between birth weight and muscle fiber number when all pigs were considered. When littermate extremes were compared by paired *t*-test smallest pigs were found to have a 15.6% reduction in total myofiber number ($P < .05$).

Discussion

These results demonstrate that birth weight is correlated with growth rate only in the early stages of pig growth, up to 25 kg in weight or 70 d of age (Figure 2). Other studies have also shown that low-birth-weight pigs grow more slowly to weaning than do their larger littermates (Campbell and Dunkin, 1982), although their feed conversion efficiencies are similar. Small pigs are able to compete less effectively for nutrition from the sow; however, growth rates and survival can be improved by separating into similar weight groups (England, 1974). This suggests that the relationship between birth weight and growth may be partly due to feed intake.

The later growth period, following d 70, seemed to be at least partially determined by the number of muscle fibers and was unaffected by birth weight (Figures 3c and 2c). This is consistent with other results suggesting that growth at this stage is determined by the pig's genotype (Blunn et al., 1953). This was also the period of fastest growth in absolute terms, with pigs increasing in weight by a mean of 890 g/d as compared with 480 g/d and 240 g/d for the second and first growth phases, respectively. These results also confirm that the same relationship exists within a strain as exists for animals of fast- and slow-growing lines, that is, a high muscle fiber number is associated with a fast growth rate (e.g., Ezekwe and Martin, 1975; Miller et al., 1975).

An additional result of this work was that feed conversion efficiency was also correlated with muscle fiber number for the third growth phase (Figure 4). Studies in runt pigs (Powell and Aberle, 1980) and genetically obese pigs (Hausman et al., 1983), both of which have a low muscle fiber number, have shown that these animals have slower and less efficient growth than pigs of normal birth weight or lean pigs. These pigs also have a greater proportion of fat in the carcass at an equivalent live weight (Robinson, 1969; Hausman et al., 1983). Additionally, strains of pigs selected for fast growth had more efficient feed conversion and contained less fat than the unselected lines (Campbell and Taverner, 1988). Fat depth has been shown to be inversely correlated with the total muscle fiber number of an "indicator" muscle (*m. flexor digiti V brevis*) in the pig (Stickland and Goldspink, 1975). This suggests that pigs with a high fiber number will have a lower amount of fat deposition, which may explain their more efficient growth. In the pig, the addition of new muscle fibers to the body of the muscle is completed in utero (Wigmore

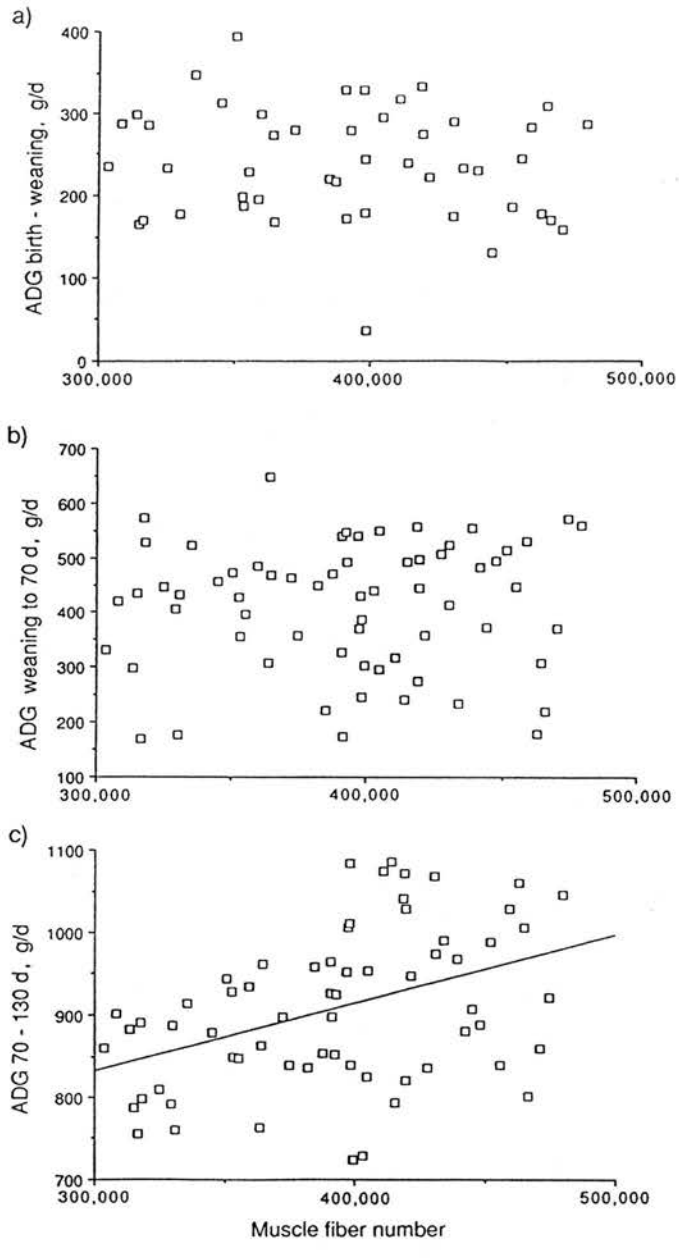

Figure 3. Average daily gain against total fiber number of semitendinosus muscle (n = 66) for (a) Phase 1, birth to 6 kg; (b) Phase 2, 6 to 25 kg; and (c) Phase 3, 25 to 80 kg. Correlation was significant for Phase 3 (r = .415, $P < .001$).

and Stickland, 1983). Postnatal growth, therefore, occurs entirely by the hypertrophy of the existing fibers. At an equivalent live weight, animals with a high fiber number will also have a smaller mean fiber diameter than animals with a low fiber number. Delivery of oxygen and nutrients to and removal of waste products from thinner vs thicker fibers may be more efficient because of the smaller diffusion distances involved (Staun, 1968).

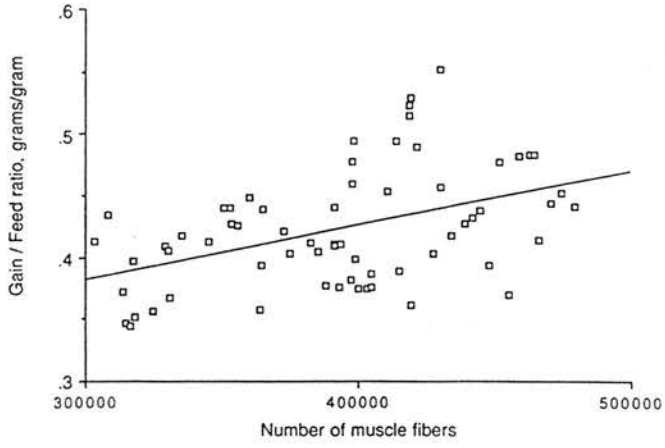

Figure 4. Gain:feed ratio against total semitendinosus fiber number (n = 66) for Phase 3 (25 to 80 kg). Correlation was significant (r = .419, $P < .001$).

It is possible that "single-site" fiber number determination, as used in this study, may not represent total fiber number. It has been suggested (Swatland and Cassens, 1972) that differences in fiber number may be due to not all fibers appearing in the plane of section because of differences in longitudinal growth of intrafascicularly terminating fibers. However, Wigmore and Stickland (1983) were unable to find intrafascicularly terminating ends of fibers in the fetal pig semitendinosus, except at the ends of the muscle, making intrafascicular fiber growth in this muscle unlikely. Furthermore, other studies (Powell and Aberle, 1981; Handel and Stickland, 1987) have failed to show an increase in semitendinosus fiber number in low-birth-weight pigs with increasing postnatal growth. Therefore, it seems likely that differences in fiber number estimates, made from the mid portion of the muscle, represent real differences in total muscle fiber number.

Although both primary fiber number and S:P ratio were positively correlated with muscle fiber numbers, only S:P ratio was directly related to ADG. Primary fiber number is known to be the determinant of interlitter variations in muscle fiber number (Dwyer and Stickland, 1991), whereas the intralitter variations in fiber number are due primarily to variations in S:P ratio caused by environmental influences. Because whole litters of animals were selected for this investigation, variations between individuals were more likely to be in secondary fiber number than in primary fiber number.

In this study there was no correlation between muscle fiber number and birth weight. Other studies have suggested, by indirect methods (Hegarty and Allen, 1978; Powell and Aberle, 1981) and by direct counting of fibers (Wigmore and Stickland, 1983; Handel and Stickland, 1987), that a low birth weight is generally associated with a reduced muscle fiber

number. These results were, however, obtained by comparing pairs of littermate extremes. Similar results were found in this study: smallest littermates had a 15.6% reduction in fiber number. However, when whole litters are considered it seems that birth weight is not a good indicator of total muscle fiber number or of postnatal growth rate. This is also seen in the phenomenon of "catch-up" growth, where pigs of low birth weight are able to achieve BW and growth rates similar to those of their littermates of heavier birth weight if they have a relatively high muscle fiber number (Handel and Stickland, 1988).

In conclusion, birth weight seems to determine the early growth rate of pigs, probably by limiting the effectiveness of competition for feed by the smaller animals. However, the number of muscle fibers seems to be a more important determinant of growth after 10 wk of age; pigs that had a high fiber number tended to grow faster and more efficiently than those that had a low fiber number.

Implications

This work demonstrates that pig birth weight is a good indicator of growth rate during the early stages of postnatal growth, but is not necessarily a determinant of growth performance to slaughter weight. Muscle fiber number may be a better indicator of potential growth during this period. Not all pigs with a high fiber number have fast and efficient growth rates. However, pigs with a low myofiber number invariably grow less well than pigs with a higher myofiber number. This indicates that a high myofiber number is a prerequisite for the potential to grow well.

Literature Cited

Blunn, C. T., G. N. Baker, and L. Hanson. 1953. Heritability of gain in different growth periods in swine. J. Anim. Sci. 12:39.

Campbell, R. G., and A. C. Dunkin. 1982. The effects of birth weight and level of feeding in early life on growth and development of muscle and adipose tissue in the young pig. Anim. Prod. 35:185.

Campbell, R. G., and M. R. Taverner. 1988. Genotype and sex effects on the relationship between energy intake and protein deposition in growing pigs. J. Anim. Sci. 66:676.

Dwyer, C. M., and N. C. Stickland. 1991. Sources of variation in myofibre number within and between litters of pigs. Anim. Prod. 52:527.

England, D. C. 1974. Husbandry components in prenatal and perinatal development in swine. J. Anim. Sci. 38:1045.

Ezekwe, M. O., and R. J. Martin. 1975. Cellular characteristics of skeletal muscles in selected strains of pigs and mice and the unselected controls. Growth 39:95.

Guth, L., and F. J. Samaha. 1970. Research note: procedure for the histochemical demonstration of actomyosin ATPase. Exp. Neurol. 28:365.

Handel, S. E., and N. C. Stickland. 1987. Muscle cellularity and birth weight. Anim. Prod. 44:311.

Handel, S. E., and N. C. Stickland. 1988. Catch-up growth in pigs: a relationship with muscle cellularity. Anim. Prod. 47:291.

Hausman, G. J., D. R. Campion, and G. B. Thomas. 1983. Semitendinosus muscle development in several strains of fetal and perinatal pigs. J. Anim. Sci. 57:1608.

Hegarty, P.V.J., and C. E. Allen. 1978. Effect of pre-natal runting on the post-natal development of skeletal muscles of swine and rats. J. Anim. Sci. 46:1634.

Luff, A. R., and G. Goldspink. 1970. Total number of fibers in muscles of several strains of mice. J. Anim. Sci. 30:891.

Miller, L. R., V. A. Garwood, and M. D. Judge. 1975. Factors affecting porcine muscle fiber type, diameter and number. J. Anim. Sci. 41:66.

Pond, W. G., H. J. Mersmann, and J.-T. Yen. 1985. Severe feed restriction of pregnant swine and rats: Effect on postweaning growth and body composition of progeny. J. Nutr. 115:179.

Powell, S. E., and E. D. Aberle. 1980. Effects of birth weight on growth and carcass composition of swine. J. Anim. Sci. 50:860.

Powell, S. E., and E. D. Aberle. 1981. Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. J. Anim. Sci. 52:748.

Robinson, D. W. 1969. The cellular response of porcine skeletal muscle to prenatal and neonatal stress. Growth 33:231.

Staun, H. 1968. Diameter and number of muscle fibres and their relation to meatiness and meat quality in Danish landrace pigs. No. 366, Beretn. fra Forsogslab., Kovenhavn, Denmark.

Stickland, N. C., and G. Goldspink. 1975. A note on porcine skeletal muscle parameters and their possible use in early progeny testing. Anim. Prod. 21:93.

Stickland, N. C., and S. E. Handel. 1986. The numbers and types of muscle fibres in large and small breeds of pigs. J. Anat. 147: 181.

Swatland, H. J., and R. G. Cassens. 1972. Muscle growth: The problem of muscle fibers with an intrafascicular termination. J. Anim. Sci. 35:336.

Wigmore, P. C., and N. C. Stickland. 1983. Muscle development in large and small pig foetuses. J. Anat. 137:235.

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Inability of muscles in the obese mouse (*ob/ob*) to respond to changes in body weight and activity

N. C. STICKLAND, R. A. L. BATT, A. R. CROOK AND C. M. SUTTON

Department of Veterinary Basic Sciences, The Royal Veterinary College, London, UK

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ABSTRACT

The fat-free carcass weight of the obese mouse (ob/ob) is generally less than that of wild-type siblings. The aim of this investigation was to examine the effect of obesity on muscle weights and histochemistry and to determine whether any effects could be eliminated when the obesity was largely prevented or reduced by limiting food intake. For 5 muscles examined the weights were significantly greater (except for biceps brachii) in the wild-type than in obese mice. Although there was a significant correlation between muscle weight (except for soleus) and body weight in the wild-type mice, no such correlation held for the obese mice. No remarkable differences between groups of mice were found in the histochemistry of the biceps brachii and soleus muscles except that fibre sizes were generally smaller in the obese mice. It is concluded that the skeletal muscles of obese mice cannot respond to the increased activity associated with prevented or reduced obesity.

Key words: Muscle histochemistry.

INTRODUCTION

The obese mouse (genotype ob/ob) is both hyperphagic and hyperlipogenic (Alonso & Maren, 1955). These features result in a body weight as much as 4 times that of wild type siblings. The difference in weight is due to massive depots of adipose tissue. The fat-free carcass weight (ffcw) is no greater or is less than that of siblings (Hollifield & Parson, 1958; Bergen et al. 1975), in spite of an adequate protein intake. The animal becomes less active (Joosten & van der Kroon, 1976) and its posture increasingly abnormal as the obesity develops. Inactivity is known to inhibit the growth of muscles (Booth, 1982). The musculature of the obese mouse could be involved in the reduced ffcw for a combination of reasons which might include either or both of the following: (1) a genetic predisposition favouring development of fat and disfavouring that of muscle: (2) atrophy of muscle through disuse, with increasing obesity. The obesity of the mouse may largely be prevented by limiting its food intake without affecting the ffcw (Robinson et al. 1975). Similarly, adult obese mice may be readily reduced in body weight. Several of the features characteristic of the animal, such as insulin resistance (Batt & Mialhe, 1966) and hypothermia (Batt & Hambi, 1982) are resolved by limiting the food intake.

Limb muscle variables were measured in both kinds of obese mice, weight limited (prevented) and weight reduced, in order to determine the relative influence of genetic and mechanical factors on muscles of locomotion. In addition, the muscles examined included one from the trunk (sternomastoid), not directly subject to the locomotor forces operating on limb muscles and thus more indicative of a direct genetic influence. The aim of this investigation was therefore to assess the effect of obesity on muscle weights and muscle histochemistry and to determine whether any effects could be eliminated when the obesity was largely prevented or reduced by limiting food intake.

Correspondence to Dr N. C. Stickland, Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, UK.

MATERIALS AND METHODS

Obese and wild type mice were produced in the ratio of 1 to 3 from parents heterozygous for the *ob* gene, in a closed colony of the BI01 strain at the Royal Veterinary College. Weaned animals were grouped and housed in polypropylene boxes at an average ambient temperature of 24 °C under artificial light (12 h light: 12 h darkness). Water and pelleted food were provided ad libitum (diets 86 and FFGM; Dixon's, Ware, UK).

Experiment 1—muscle weights

Ad libitum fed, male obese animals (n = 9) and their wild-type litter mates (n = 9) were weighed and decapitated at 265 d at which age the growth plateau in the wild type had been attained. The following muscles were removed from both sides of the animal and weighed: biceps brachii, rectus femoris, tibialis cranialis, soleus and sternomastoid. A further group of male obese animals (n = 5) which had been limited from weaning to that amount of food eaten by their wild-type litter mates (up to 3.5 g daily) were treated similarly.

Experiment 2—muscle histochemistry

Three groups of animals were selected, similar to those in Experiment 1 except that they were all female. A 4th group of obese animals were allowed to grow and were subsequently *reduced* in body weight from a mean value of 94.5 g to 58.0 g by limiting the food intake to a minimum of 2.2 g daily over a period of 78 d. (The difference in final body weight for the amount of food fed to obese mice *prevented* from growing and those *reduced* in body weight would indicate the reluctance with which their fat depots are depleted). All 4 groups of animals (total n = 38) were weighed and decapitated. The biceps brachii and soleus muscles were removed, weighed and rapidly frozen in dichlorodifluoromethane (Arcton, ICI Ltd), cooled to its melting point of −158 °C in liquid nitrogen. Transverse cryostat sections (10 μm) were cut from each muscle and serial sections were reacted for acid and alkali-stable myosin ATPase (Guth & Samaha, 1970), succinic dehydrogenase (Nachlas et al. 1957) and glycogen phosphorylase (Takeuchi, 1956). These sections were used to assess the number, proportion and size of constituent muscle fibres in each muscle. Fast fibres were detected by alkali stable myosin ATPase reaction, slow fibres by acid stable myosin ATPase, oxidative fibres by succinic dehydrogenase activity and glycolytic fibres by glycogen phosphorylase activity. The use of serial sections enabled muscle fibres in the mouse to be classified as fast oxidative glycolytic (FOG), fast glycolytic (FG) or slow oxidative (SO). For each muscle the total number of fibres were counted as was the proportion of each fibre type. Mean cross-sectional areas were based on measurements of 100 fibres of each type for each muscle. Muscle fibres were measured using a Seescan image analysis system (Seescan plc, Cambridge).

For both experiments differences between parameters were assessed by analysis of variance followed by a Neuman-Keul test with a probability of less than 5% being deemed significant.

RESULTS

Experiment 1—muscle weights

The mean body weight of ad libitum fed, obese mice was more than twice that of their wild-type littermates (90 g compared with 40 g; Fig. 1). Pair feeding obese with wild-type mice from weaning largely prevented this difference although excessive adipose tissue was still evident in these pair-fed animals. The difference was not reflected in the muscle weights (Fig. 2). The mean value for all 5 of the muscles examined was greater (12–33%; $P < 0.05$) in wild-type mice than in either of the obese groups. The exception was the biceps brachii for which only the limit fed obese muscles were different from the wild type.

There was a significant correlation between the weight of each muscle and body weight within the wild-type group (except for the soleus) (see Table). However, no such correlation held for the obese mice.

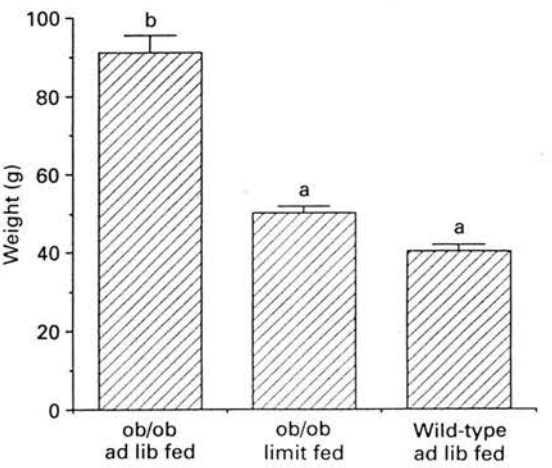


Fig. 1. Body weights of the male mice used in Experiment 1. Error bars represent S.E.M.S. Columns with different letters are significantly different at $P < 0.05$.

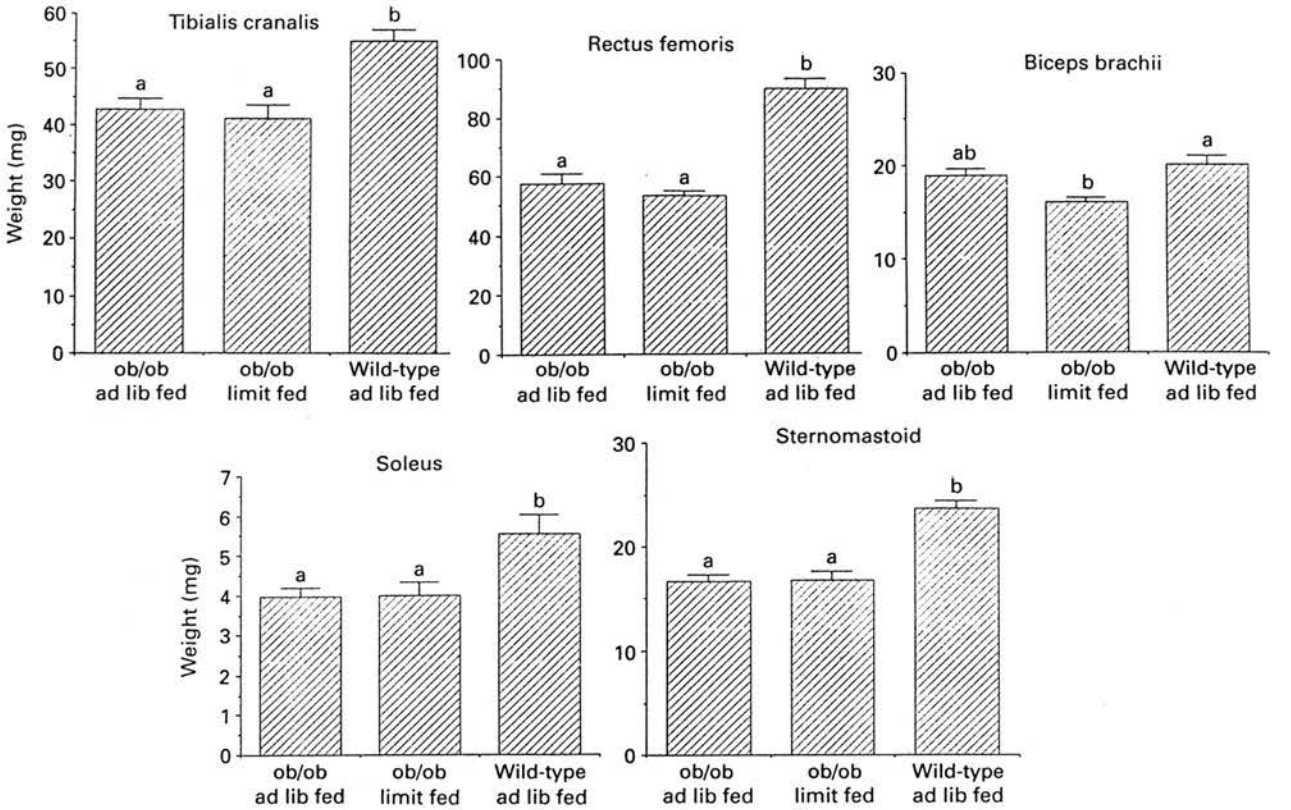


Fig. 2. Muscle weights of the male mice used in Experiment 1. Error bars represent S.E.M.S. For each muscle, columns not sharing the same letters are significantly different at $P < 0.05$.

Table. *Correlation coefficients of muscle weight against body weight*

	Tibialis cranialis	Soleus	Rectus femoris	Biceps brachii	Sterno-mastoid
Wild-type ad lib fed (n = 9)	0.844**	0.510	0.738*	0.713*	0.672*
ob/ob ad lib fed (n = 9)	0.211	0.084	0.213	0.466	0.037
ob/ob limit fed (n = 5)	0.056	0.481	0.287	0.769	0.552

* Significant at $P < 0.05$; ** significant at $P < 0.01$.

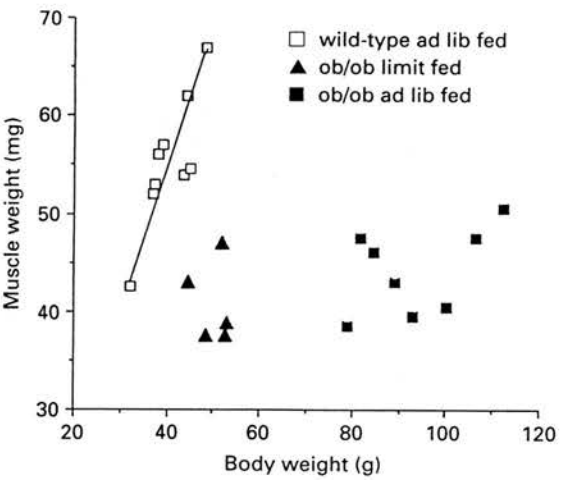


Fig. 3. Correlation of weight of tibialis cranialis against body weight for the 3 groups of mice used in Experiment 1.

Muscle weights (Fig. 2) and their lack of correlation with body weight (Table) remained unaltered in the obese mouse following the prevention of the excessive body weight, through pair feeding. Figure 3 shows the relationship between muscle weight (tibialis cranialis) and body weight for all 3 groups of mice.

Experiment 2—muscle histochemistry

Body weights were similar to those of the equivalent groups in experiment 1 (Fig. 4). The additional, weight-reduced group of obese mice weighed 58 g. This was 21 g (57%) heavier than wild-type mice and 12 g (26%) heavier than those prevented from gaining weight. The mean total fibre number in soleus ranged from 610 to 750 between all groups. No one group was significantly different from any other (Fig. 5). There was also no significant intergroup difference in the total fibre numbers for biceps brachii (results not shown).

The histochemistry demonstrated 2 main fibre types in the soleus, namely FOG and SO fibres. The

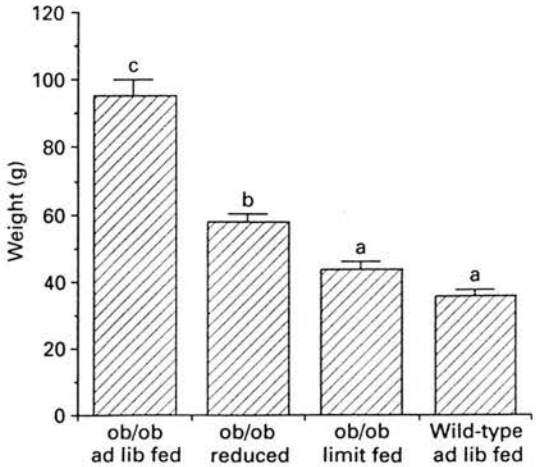


Fig. 4. Body weights of the female mice used in Experiment 2. Error bars represent S.E.M.S. Columns with different letters are significantly different at $P < 0.05$.

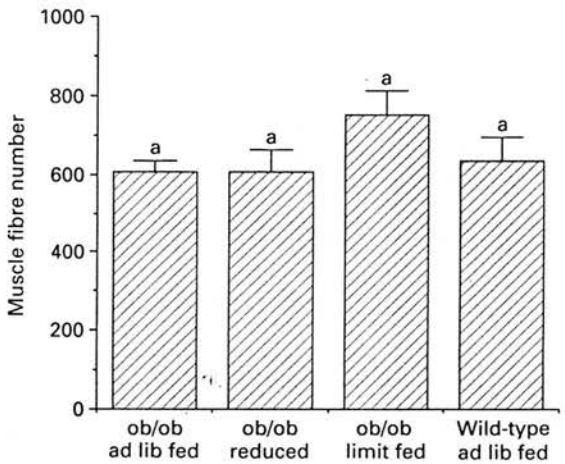


Fig. 5. Total number of muscle fibres in soleus of the mice used in Experiment 2. Error bars represent S.E.M.S. Columns with different letters are significantly different at $P < 0.05$.

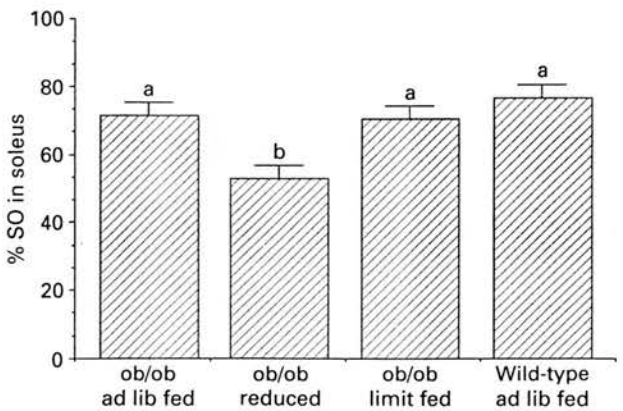
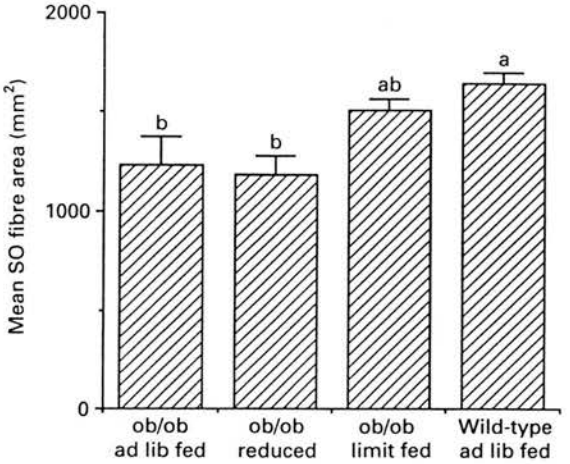


Fig. 6. Percentage of slow oxidative (SO) fibres in soleus of the mice used in Experiment 2. Error bars represent S.E.M.S. Columns with different letters are significantly different at $P < 0.05$.



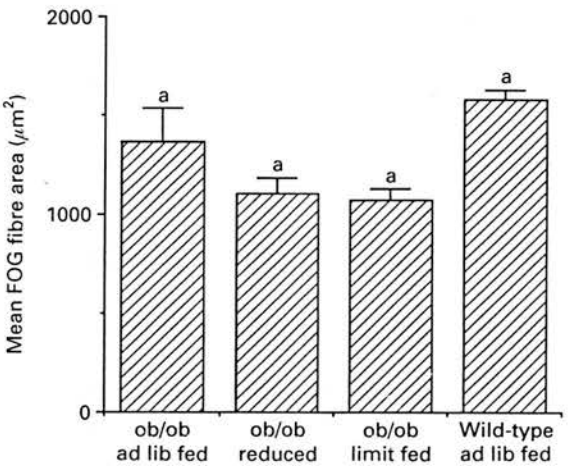


Fig. 7. Mean cross-sectional area of slow oxidative (SO) fibres and fast oxidative, glycolytic (FOG) fibres in soleus of mice used in Experiment 2. Error bars represent S.E.M.S. Columns not sharing the same letters are significantly different at $P < 0.05$.

percentage of SO fibres was significantly lower in the weight-reduced obese mice (Fig. 6) and that of the FOG fibres therefore higher. Although the mean cross-sectional areas of both FOG and SO fibres in soleus appeared lower in all groups of obese mice than in the wild-type mice (Fig. 7), the differences were only significant for the SO fibres of ad libitum fed and weight-reduced obese mice.

For biceps brachii the histochemistry demonstrated that FOG and FG were the main fibre types. No significant differences were found between groups of mice in either fibre type proportions or in mean size of fibre types.

DISCUSSION

A lower ffcw in the face of pronounced obesity has frequently been recorded for the obese mouse. The difference has been particularised for various muscles (e.g. Almond & Enser, 1984; Purchas et al. 1985). This

present study confirms the lower weights of muscles in the obese mouse. The difference was considerable (as much of 33% for rectus femoris), compared with wild-type siblings. A small group of 3 ad libitum fed obese female mice had similar muscle weights to those of their male counterparts (Batt, unpublished observation), indicating that the underdevelopment is not due to reduced androgens. Immobilisation is known to result in muscle atrophy (Booth, 1982), and heavier obese mice are markedly immobile (Mayer, 1953). The pelvic limbs may not reach the floor due to the width of the trunk and the animal may be observed dragging its body forward by the use of its fore limbs. Immobility may therefore be responsible for lower muscle weights in the ad lib obese mice and for lack of significant correlation in the group between the weights of muscles of locomotion and body weight. In the wild-type group a significant correlation held for 4 of the 5 muscles examined. The low significance for the 5th muscle, soleus, may be attributed to its small size, such that errors in dissection and muscle cleaning would be magnified. The low muscle weights were not altered in those obese mice prevented dietarily from attaining excessive body weight. This is despite the fact that all mice were less than 75 g, a weight at which obese mice are quadrupedal. This is evidence for the low muscle weights of ad lib obese mice not being due to immobilisation. The weight of each muscle and its relationship with body weight remained unchanged following control of the obesity from 125% down to 25% in excess of wild-type body weight. Parrott & Batt (1980) have shown the spontaneous activity rate in such pair-fed obese animals to be the same as that of wild type siblings, and much greater than that of ad lib fed obese mice (Batt, 1983). The experimental protocol used in these behavioural studies was the same as that used in the current study. However, even when the activity of obese mice is increased, the ffcw still remains low (Welton et al. 1973). Incidentally, the adipose tissue was reduced but still excessive in these pair-fed animals compared with their wild type siblings (Alonso & Maren, 1955). Apart from the evidence in the limb muscles, the sternomastoid (a flexor of the head and neck on the trunk) is also considerably lower in weight (by 33%) than in the wild type. There is a possibility that the pair-fed obese animals could not undergo muscle hypertrophy because of the reduced food intake but feeding levels were, of course, not less than for the wild type controls. Overall the results suggest an intrinsic incapacity in the muscles of the obese mouse to respond to changes in body weight and activity. An exception to this may be the biceps brachii. The considerable work involved for the obese mouse in dragging its body forward may account for the greater relative weight of biceps brachii in obese mice compared with the other muscles. Thus limiting the body weight of obese mice resulted in a significant reduction in the weight of this muscle. The jaw muscle of obese mice (masseter) may also have some capacity for hypertrophy. The animal is hyperphagic, consuming 35% more pelleted food than wild-type siblings in the present colony (Batt et al. 1978). Associated with this increase in work, the masseter was not significantly different in weight from that of wild-type siblings (M. Robins, personal communication).

The considerable difference in muscle weights was not associated with obvious histological or histochemical changes. Total fibre number in soleus and biceps was similar in all 4 groups of mice. In soleus, both fibre types were well represented. The proportion of SO fibres appeared to be lower in the weight-reduced obese muscle but, as the proportion in the other 2 obese groups was similar to the wild type, it is a difficult result to explain. However, weight reduced and weight limited obese mice, though of similar body weight, may not be strictly comparable preparations, as the former group will exhibit greater hunger and activity. Even so, this difference in activity does not explain the difference seen in the proportion of SO fibres. The cross-sectional areas of constituent muscle fibres in soleus were generally less in the obese mice although this was only significant for the SO fibres in the ad lib and weight-reduced obese mice. The tendency for smaller fibres in the obese mice is a reflection of the lower soleus muscle weights in these animals. However, the difference (up to 25%) in fibre areas does not account for the 33% difference in soleus muscle weights. It is relevant, however, that the long bones are reduced in length. Specifically the femur is up to 10% reduced (M. Robins, personal communication). Applying a similar reduction in length to the soleus would account for a proportion of the muscle weight difference. Possible differences in muscle connective tissue between groups might also have emphasised the difference. In biceps brachii there was no difference between groups in the proportions or sizes of fibre types, which is in agreement with Almond & Enser (1984) and which reflects (for fibre sizes) the lack of clear differences in weights of biceps brachii (Fig. 2). Taken as a whole the results show no evidence that muscles of obese mice are compromised with respect to their contractile capacity but rather that they exhibit a reluctance to hypertrophy.

The failure of the muscles of obese mice to respond to recognised stimulating and inactivating forces indicates that their reduced weight is due to a more direct effect of the recessive gene rather than consequences of the obesity. The gene's retarding influence on growth involves also the associated skeletal system in the overall reduced ffcw. The systemic control of somatic growth is determined ultimately by the discharge of somatotropin-releasing-hormone from the arcuate nucleus and the immediate surrounding area of the hypothalamus (Jacobowitz et al. 1983). Satiety is governed by the ventromedial nucleus of the hypothalamus immediately adjacent to the arcuate nucleus (Hetherington & Ranson, 1942). A reduced sensitivity of the nucleus to glucose has been reported for the obese mouse (Baile et al. 1970) as well as a feeding behaviour characteristic of animals with damage to the nucleus (Fuller & Jacoby, 1955). Hyperlipogenic obesity in combination with retarded growth has been produced in the weanling rat by electrolytic lesions in the hypothalamus (Kennedy, 1966). The 2 features were not dissociable nor accompanied by hyperphagia (Han et al. 1965). The animal's concomitant sterility is probably hypothalamic in origin (Batt et al. 1982) and again it is noteworthy that the site of origin of LHRH (the median eminence) is confluent with the arcuate nucleus. Moreover, of the numerous abnormalities present in the obese mouse, it is the 3 functions governed by these nuclei that do not respond to controlling the food intake. All 3 functions determine growth of their respective tissues. Their degree of impairment is not extreme, indicating that the affected gene is involved in a polygenic effect. Other genes having a similar effect to *ob* have been well documented for the mouse (Bray & York, 1979). The combination of obesity, reduced ffcw and sterility also occurs in the rat homozygous for the recessive gene, *fa* (fatty) in which the reduced muscle weight (gastrocnemius) is accentuated by pair-feeding, in contrast to the obese mouse (Shapira et al. 1980). Studies on inherently obese laboratory animals are relevant to the strikingly similar combination of features characteristic of the Prader Willi syndrome in man (Robinson et al. 1991).

At the tissue level there are many factors which are known to affect muscle growth and several are implicated in the obese mouse. Low circulating levels of growth hormone, as found in the obese mouse (Sinha et al. 1975), are known to impair muscle growth (Buul-Offers, 1983) probably through effects on insulin-like growth factor 1 (Zapf et al. 1981). Growth hormone may also be implicated in the apparent transition to a more oxidative metabolism in obese muscles (Almond & Enser, 1984). High levels of circulating corticosteroids which are found in obese mice (Dubuc, 1977) are also known to impair muscle growth (Goldberg et al. 1980). Furthermore, it is possible that the reduced levels of androgens characteristic of obese mice may adversely affect the growth of specific muscles, said to be sensitive to these steroid hormones (Kelly et al. 1985). However, in this histochemical study all mice used were female and there seems to be no evidence in the literature for an oestrogen effect on biceps brachii or soleus muscles. Interestingly, beta blocking drugs are known to aggravate the problem of obesity in *ob/ob* mice (Batt et al. 1978). This may point to low levels of noradrenaline at the muscle fibre level. Although raised hypothalamic noradrenaline levels have been reported in the obese mouse and these levels persist with limit feeding (Oltmans et al. 1976), it has been suggested that release of noradrenaline into the circulation may be impaired (Batt et al. 1982).

The results of this present investigation have shown that obese mouse muscle, although not showing significant intrinsic abnormalities in physiological properties, does exhibit an inability to hypertrophy in response to functional demand.

REFERENCES

ALMOND RE, ENSER M (1984) A histochemical and morphological study of skeletal muscle from obese hyperglycaemic ob/ob mice. *Diabetologia* **27**, 407–413.

ALONSO LG, MAREN TH (1955) Effect of food restriction on body composition of hereditary obese mice. *American Journal of Physiology* **183**, 284–290.

BAILE CA, HERRERA MG, MAYER J (1970) Ventromedial hypothalamus and hyperphagia in hyperglycemic mice. *American Journal of Physiology* **218**, 857–863.

BATT RAL (1983) Decreased food intake in response to cholecystokinin (pancreozymin) in wild-type and obese mice (genotype ob/ob). *International Journal of Obesity* **7**, 25–29.

BATT RAL, MIALHE P (1966) Insulin resistance of the inherently obese mouse-ob/ob. *Nature* **212**, 289–290.

BATT RAL, WILSON CA, TOPPING DL (1978) Potentiation of hyperphagia and relief of hypothermia in the genetically obese mouse (genotype, ob/ob). *International Journal of Obesity* **2**, 303–307.

BATT RAL, EVERARD DM, GILLIES G, WILKINSON M, WILSON CA, YEO TA (1982) Investigation into the hypogonadism of the obese mouse (genotype, ob/ob). *Journal of Reproduction and Fertility* **64**, 363–371.

BATT RAL, HAMBI M (1982) Development of hypothermia in obese mice (genotype ob/ob). *International Journal of Obesity* **6**, 391–397.

BERGEN W, KAPLAN M, MERKEL RA, LEVEILLE GA (1975) Growth of adipose and lean tissue mass in hind limbs of genetically obese mice during preobese and obese phases of development. *American Journal of Clinical Nutrition* **28**, 157–161.

BOOTH FW (1982) Effect of limb immobilisation on skeletal muscle. *Journal of Applied Physiology* **52**, 1113–1118.

BRAY GA, YORK DA (1979) Hypothalamic and genetic obesity in

experimental animals: an autonomic and endocrine hypothesis. *Physiological Reviews* **59**, 719–809.

BUUL-OFFERS S VAN (1983) Hormonal and other inherited growth disturbances in mice with special reference to the Snell dwarf mouse. A review. *Acta Endocrinologica* **103** (Suppl. 258).

DUBUC P (1977) Basal corticosterone levels of young ob/ob mice. *Hormone and Metabolic Research* **9**, 95–97.

FULLER JL, JACOBY GA JR (1955) Central and sensory control of food intake in genetically obese mice. *American Journal of Physiology* **183**, 279–283.

GOLDBERG AL, TISCHLER M, DE MARTINO C, GRIFFIN G (1980) Hormonal regulation of protein degradation and synthesis in skeletal muscle. *Federation Proceedings* **31**, 31–36.

GUTH L, SAMAHA FJ (1970) Research note: procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28**, 365–367.

HAN PW, LIN C-H, CHU K-C, MU J-Y, LIU A-C (1965) Hypothalamic obesity in weanling rats. *American Journal of Physiology* **209**, 627–631.

HETHERINGTON AW, RANSON SW (1942) The relation of various hypothalmic lesions to adiposity in the rat. *Journal of Comparative Neurology* **76**, 475–499.

HOLLIFIELD G, PARSON W (1958) Body composition of mice with goldthioglucose and heredity obesity after weight reduction. *Metabolism* **7**, 179–183.

JACOBOWITZ DM, SCHULTE H, CHROUSOS GP, LORIAUX DL (1983) Localization of GRF-like immunoreactive neurones in the rat brain. *Peptides* **4**, 521–524.

JOOSTEN HFP, VAN DER KROON PHW (1976) Growth pattern and behavioural traits associated with the development of the obese-hyperglycemic syndrome in mice (ob/ob). *Metabolism* **23**, 1141–1147.

KELLY A, LYON SG, GAMBKE B, RUBINSTEIN N (1985) Influences of testosterone on contractile proteins of the guinea pig temporalis muscle. *Advances in Experimental Medicine and Biology* **182**, 155–168.

KENNEDY GC (1966) Food intake, energy balance and growth. *British Medical Bulletin* **22**, 216–220.

MAYER J (1953) Decreased activity and energy balance in the hereditary obese-diabetes syndrome of mice. *Science* **117**, 504–505.

NACHLAS MM, TSOU K, DE SOUSA E, GIENG C, SELIGMAN AM (1957) Cytochemical demonstration of succinic dehydrogenase by the use of new p nitro phenyl substituted ditetrazole. *Journal of Histochemistry and Cytochemistry* **5**, 420–436.

OLTMANS GA, LORDEN JF, MARGULES DL (1976) Effects of food restriction and mutation on central catecholamine levels in genetically obese mice. *Pharmacology, Biochemistry and Behaviour* **5**, 617–620.

PARROTT RF, BATT RAL (1980) The feeding response of obese mice (genotype, ob/ob) and their wild-type littermates to cholecystokinin (pancreozymin). *Physiology and Behaviour* **24**, 751–753.

PURCHAS RW, ROMSOS DR, ALLEN RE, MERKEL RA (1985) Muscle growth and satellite cell proliferative activity in obese (ob/ob) mice. *Journal of Animal Science* **60**, 644–651.

ROBINSON DW, HODGSON D, BRADFORD GE, ROBB J, PETERSON DW (1975) Effects of dietary restriction and fasting on the body composition of normal and genetically obese mice. *Journal of Animal Science* **40**, 1058–1062.

ROBINSON W, BOTTANI A, YAGANE X, BALAKRISHMAN J, BINKERT F, MÄCHLER M, et al. (1991) Molecular, cytogenetic and clinical investigations of Prader-Willi syndrome patients. *American Journal of Human Genetics* **49**, 1219–1234.

SHAPIRA JF, KIRCHER I, MARTIN RJ (1980) Indices of skeletal muscle growth in lean and obese zucker rats. *Journal of Nutrition* **110**, 1313–1318.

SINHA YN, SALOCKS CB, VANDERLAAN WP (1975) Prolactin and growth hormone secretion in chemically induced and genetically obese mice. *Endocrinology* **97**, 1386–1393.

TAKEUCHI T (1956) Histochemical demonstration of phosphorylase. *Journal of Histochemistry and Cytochemistry* **4**, 84.

WELTON RF, MARTIN RJ, BAUMGARDT BR (1973) Effects of feeding and exercise regimens on adipose tissue glycerokinase activity and body composition of lean and obese mice. *Journal of Nutrition* **103**, 1212–1219.

ZAPF J, WALTER H, FROESCH ER (1981) Radioimmunological determination of IGFI and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *Journal of Clinical Investigation* **68**, 1321–1330.

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Muscle at birth in mice selected for large and small body size

S. C. BROWN AND N. C. STICKLAND

Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London, UK

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ABSTRACT

Selection for divergent body weight at 6 wk of age in the Q strain mouse has produced large (QL) and small (QS) mice which differ 2-fold in their adult body weight. The purpose of this investigation was to identify some of the cellular mechanisms which underlie the early divergence in size between the 2 lines. At birth, QL mice (for similar litter sizes) were 28% heavier and 6% longer than QS mice. This was reflected by measurements of longitudinal bone length which were greater in QL (tibia 6.2%, humerus 4.2%) compared with QS mice. Fibre number was found to be 18 and 17% greater in the biceps brachii and soleus muscles respectively of the QL mice. It was concluded that this was not a consequence of any alteration in the ratio of developing secondary to primary myofibres in either muscle. Fibre cross-sectional areas were only significantly different between the QL and QS for the soleus muscle, which might be explained by the relatively greater divergence in the length of its supporting bone (tibia) between the QL and QS compared with the humerus. Estimates of nuclear number showed that there were significantly more nuclei in biceps brachii muscle of QL than in the QS mice which could be attributed to the difference in fibre number, although no such differences were found for the soleus muscle. There was no apparent alteration in the proportion of nuclei found within the fibres of the biceps muscle. Overall the results indicate that selection in this situation has acted through the normal cellular processes of growth.

Key words: Q strain mice; growth.

INTRODUCTION

Selection for divergent body weight at 6 wk in the Q strain mouse has produced animals which differ 2-fold in their adult body weight (Falconer, 1973). Furthermore, it has been shown that this selection procedure produces the correlated response of an increase in growth rate both before and after weaning (Rucklidge, 1981). Whilst analyses of the body composition of these mice seem to indicate that the 2 lines show similar patterns of postnatal growth (Rucklidge, 1981), quantitative changes in total DNA and protein:DNA ratios fail to define the cellular mechanisms which underlie genetically determined differences in muscle mass.

We have shown that primary myotube number is significantly increased within the biceps brachii of QL compared with QS mice (Brown & Stickland, unpublished results). These observations, in the absence of any detectable difference in nuclear density, suggested that selection had altered the rate of myoblast proliferation thereby affecting the number of cells available for fusion. However, there is increasing evidence to suggest that primary and secondary myotubes are derived from separate myogenic lineages (Miller & Stockdale, 1986), each of which could respond differently to selection pressure. Indeed previous work in the pig seems to indicate that primary and secondary generation myotubes contribute unequally to genetically determined alterations in fibre number (Handel & Stickland, 1984).

Fibre number is not the only variable altered by selection for divergent body weight; alterations in muscle length and fibre transverse sectional area are also evident in mature mice (Hooper, 1978). Aspects of fibre size may, however, be very responsive to environmental influences and functional demand. Many of these influences are minimal up to the time of

Correspondence to Dr S. C. Brown, Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF.

birth such that any fibre size differences seen at birth may be more indicative of direct genetic effects. The present investigation therefore concentrates on the relative importance of fibre and nuclear number, fibre transverse section area and fibre length in the newborn QL and QS, with the aim of identifying some of the cellular mechanisms underlying the early divergence in size between the 2 lines.

MATERIALS AND METHODS

Relationships between litter size and pup mean body weight in the 2 lines were assessed on the basis of measurements made from 11 QL and 29 QS newborn litters. Crown–rump length and body weight were specifically measured in 24 QL and 44 QS randomly selected littermates. For analyses of muscle transverse sectional area, fibre number and fibre size, the largest, average and smallest littermates, on the basis of weight, were selected at birth from each of 5 litters of high body weight (QL) and 5 litters of low body weight (QS).

Animals were killed by decapitation and all skinned limbs were initially fixed in the resting position for 15 min in 0.1 M sodium cacodylate buffer pH 7.3 containing 2% glutaraldehyde. Biceps brachii was dissected from the forelimb, fixed for a further 3 h in 2% glutaraldehyde, washed in cacodylate buffer for 1 h, fixed for 1.5 h in an aqueous solution containing 0.6% osmium tetroxide and 0.4% potassium ferrocyanide (Aguas, 1982), washed in distilled water, dehydrated through graded acetone up to 100%, infiltrated and embedded in Araldite resin (CY212) which was polymerised at 60 °C for 4–6 d. Lower hind limbs were processed intact but skinned due to the difficulty in dissecting the newborn soleus muscle.

Semithin sections (1 μm) were taken from the midbelly of the biceps brachii muscles and stained with 1% methylene blue. The midbelly region of each soleus muscle was assessed by sequential sectioning through a sample fore and hindlimb until the muscle displayed its maximal girth, at which point the relative position down the limb was noted together with the relative sizes of adjacent muscles. Subsequent limbs were then sectioned accordingly. In the absence of serial sectioning through each hindlimb this method was deemed to be the most accurate way of assessing the midbelly region of each soleus muscle. Ultrathin transverse sections (90 nm) taken from the same region of each muscle as the semithin (1 μm) sections were collected on 100 mesh copper grids. Sections were then stained in 1% uranyl acetate (40 min) followed by Reynolds (1963) lead citrate (5 min), and examined using a JEOL 1200X electron microscope operating at an accelerating voltage of 80 kV.

Since individual fibres were difficult to distinguish in the centre of biceps brachii with the light microscope, low power (×250) electron micrographs of randomly selected areas were used to determine fibre and nuclear density. Total fibre and nuclear numbers were then calculated using total transverse sectional area of the muscles measured by light microscopy. Whilst this method may lead to inaccuracies as a consequence of the differential rate of stretching in the semithin and ultrathin sections, these were assumed to be less than those which might result from direct fibre counts on semithin sections. Moreover, any error incurred would be expected to be the same within the QL and QS. The proportion of nuclei in the myofibres was evaluated on electron micrographs of more than 200 fibres taken at ×800. The relative proportion of satellite to myofibre nuclei has previously been shown not to be significantly different between these 2 lines of mice (Brown & Stickland, 1993). Satellite cell nuclei were therefore not counted separately and due to their close apposition to the muscle fibre were included in the counts of myofibre nuclei. Capillary nuclei were not included in any estimations of nuclear number.

Fibre transverse sectional area measurements in biceps brachii were carried out on low power electron micrographs taken at ×400 and ×800 which were calibrated using a grid ruled at 1200 lines/mm. Measurements of areas of at least 200 fibres from each muscle were made using an Apple IIe computer (VIDS 11 analytical measuring system). Measurements of fibre transverse sectional area in soleus were carried out on light microscope images of 1 μm semithin sections using a Kontron MOP interactive image analysis system; 200 fibres were measured across the entire girth of each muscle.

Ossified bone length was assessed in the fore and hindlimb of randomly selected newborn QL (20 forelimbs and 17 hindlimbs) and QS (36 forelimbs and 19 hindlimbs) using microradiography. Radiographs of limbs previously fixed in formalin were made using a Hewlett Packard Faxitron x-ray machine with an exposure of 40 kV, 2.5 mA for 15 min onto Kodak Spectroscopic plates. Development was carried out in D19 (Kodak) for 10 min. Negatives were subsequently printed and the length of ossified bone measured using the Kontron MOP Videoplan image analysis system. A scale bar on the original radiograph provided the basis for calibration. Whilst this measurement did not represent the actual bone length, it was considered the most accurate assessment of this variable in view of the size of the specimens.

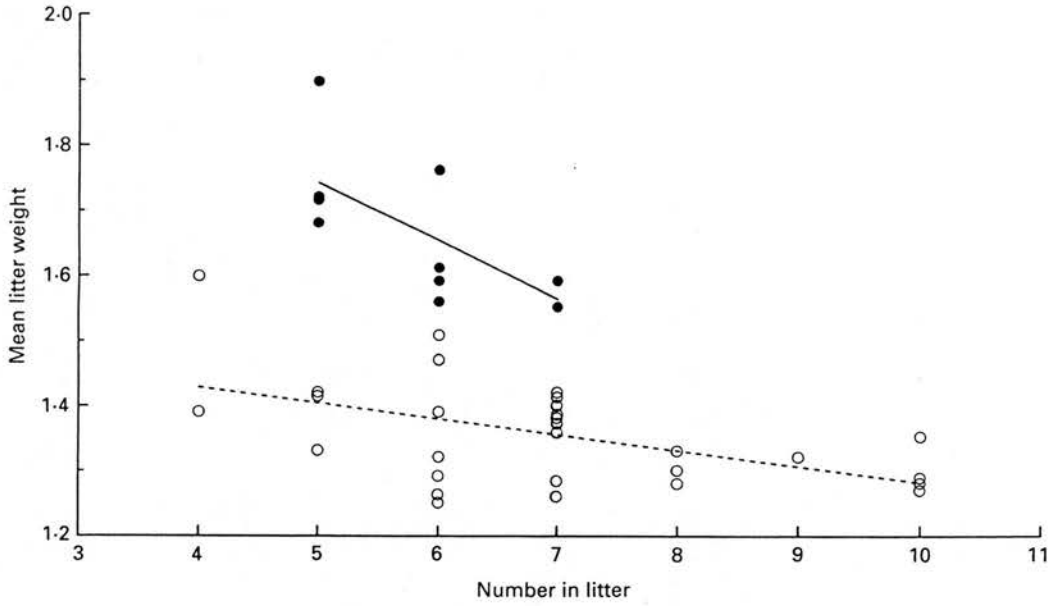

Fig. 1. Relationship between mean pup weight and number in the litter in the QL and QS mice. The regression equations for the QL and QS were $y = 2.19 - 0.09x$ ($r^2 = 0.5$) and $y = 1.5 - 0.02x$ ($r^2 = 0.3$), respectively. ●—●, QL; ○---○, QS.

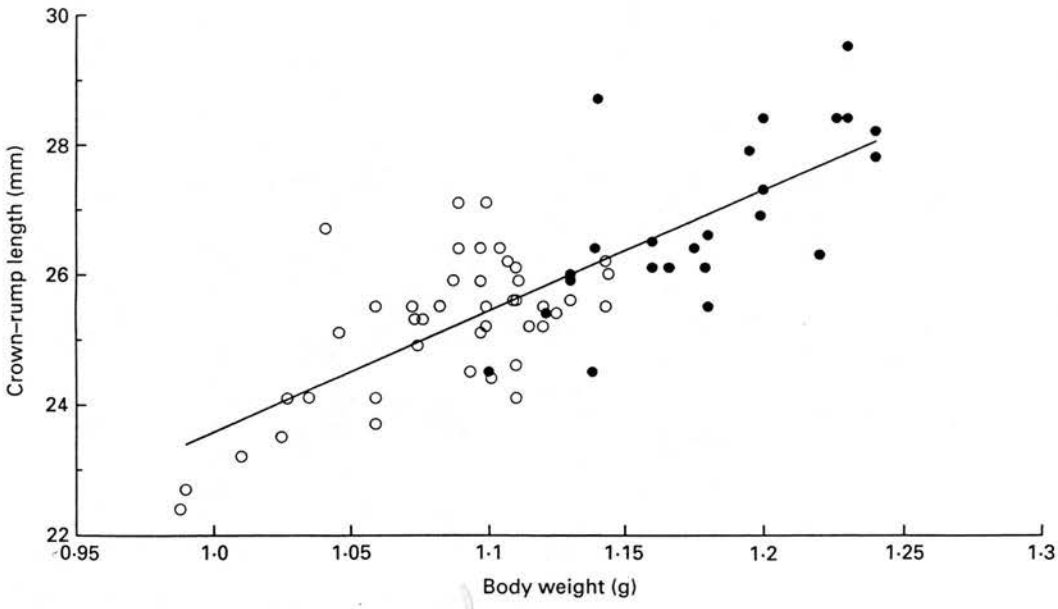

Fig. 2. Relationship between crown–rump length and body weight in the newborn QL and QS mice. The regression equations for the QL and QS were $y = -0.8 + 23.4x$ ($r^2 = 0.5$) and $y = 5.6 + 18.06x$ ($r^2 = 0.4$), respectively. ●, QL; ○, QS; —, QL/QS.

RESULTS

Birth weight in the QL and QS mice

There appeared to be no significant difference in gestational age between the QL and QS. Figure 1 shows the relationship between litter size and mean pup weight for 11 QL and 29 QS litters.

A comparison of randomly selected QL (n = 24) and QS (n = 44) showed that both crown–rump length and body weight were significantly different between the 2 lines at birth ($P \leqslant 0.001$). However, in view of the above correlation between litter size and mean litter weight, comparisons of crown–rump length and weight were carried out on 4 litters matched for litter size from each of the lines. These results confirmed the findings on randomly selected individuals ($P \leqslant 0.01$). Crown–rump length was increased by 6% in the QL relative to the QS, whilst the corresponding increase in body weight was 28%.

Crown–rump length in newborn QL and QS mice

Figure 2 shows the relationship between crown–rump length and body weight in newborn QL and QS mice.

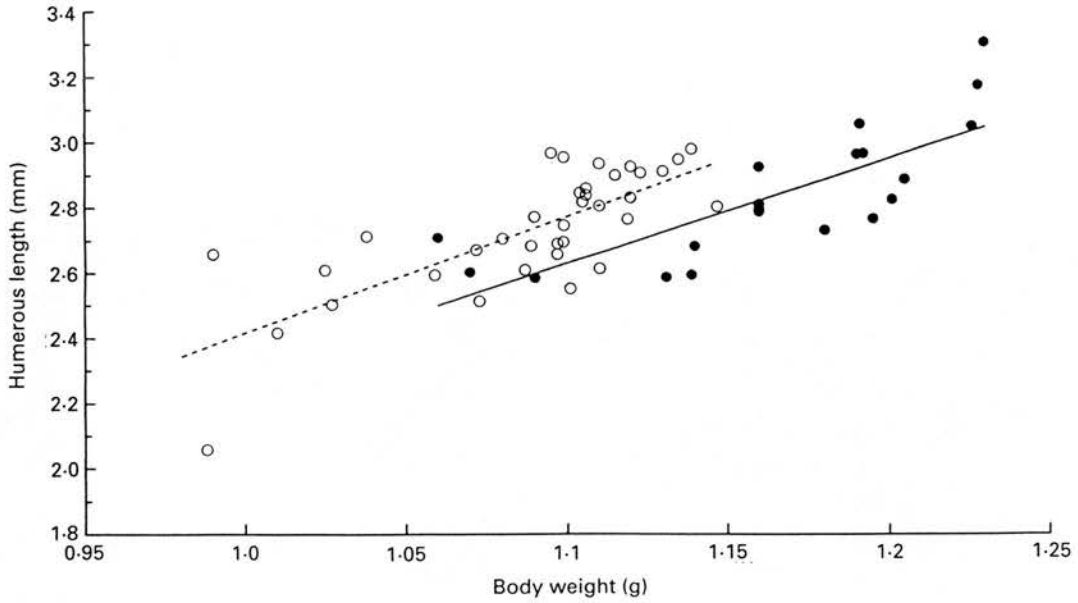

Fig. 3. Relationship between the length of the humerus and body weight in the newborn QL and QS mice. The regression equations for the QL and QS were $y = 0.9 + 3.2x$ ($r^2 = 0.6$) and $y = -1.11 + 3.5x$ ($r^2 = 0.6$), respectively. ●—●, QL; ○---○, QS.

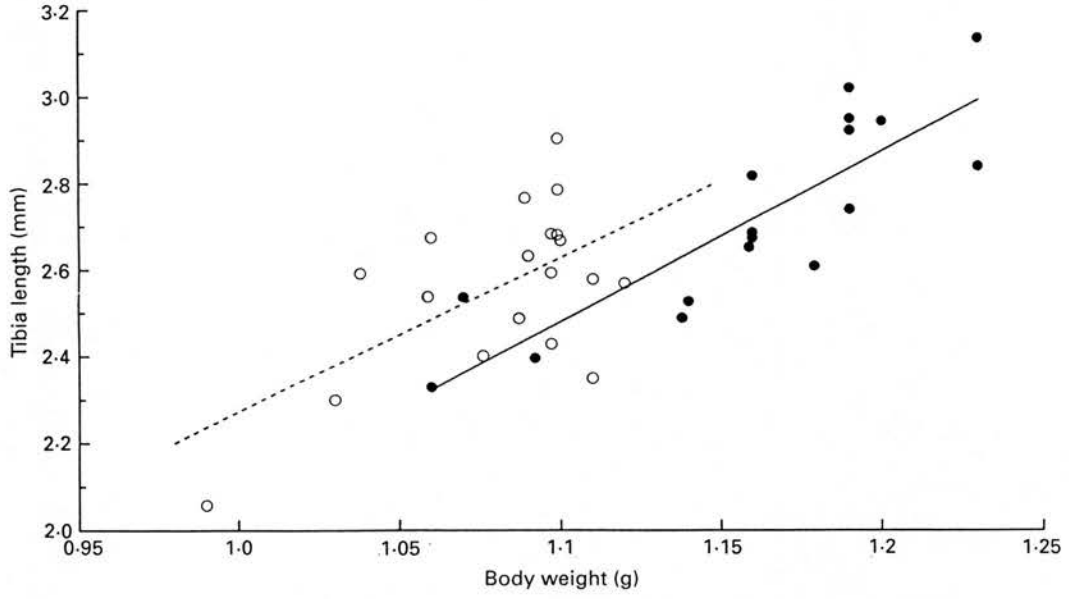

Fig. 4. Relationship between the length of the tibia and body weight in the newborn QL and QS mice. The regression equations for the QL and QS were $y = -1.8 + 3.93x$ ($r^2 = 0.7$) and $y = -1.3 + 3.6x$ ($r^2 = 0.4$), respectively. ●—●, QL; ○---○, QS.

These 2 variables were significantly associated in the QL ($P \leqslant 0.001$) and the QS ($P \leqslant 0.001$) mice (t test of the regression coefficients). A statistical comparison of the 2 lines showed no significant difference either in the slope or intercept.

Bone length in newborn QL and QS mice

Measurements of ossified bone length in the upper forelimb and lower hindlimb of the QL and QS mice were performed in order to obtain an approximation of muscle length in biceps brachii and soleus.

A comparison of the length of the humerus in randomly selected newborn QL and QS mice showed there to be a significant difference between the lines ($P \leqslant 0.05$). The percentage increase in the QL relative to the QS was 4.2. Figure 3 illustrates the relationship between the length of the humerus and body weight in the newborn QL and QS mice. These 2 variables were significantly associated in the QL ($P \leqslant 0.001$) and the QS ($P \leqslant 0.0001$) mice (t tests of the regression coefficients). A statistical comparison of these 2 lines with respect to slope and intercept indicated a significant difference in the intercept only ($P \leqslant 0.002$).

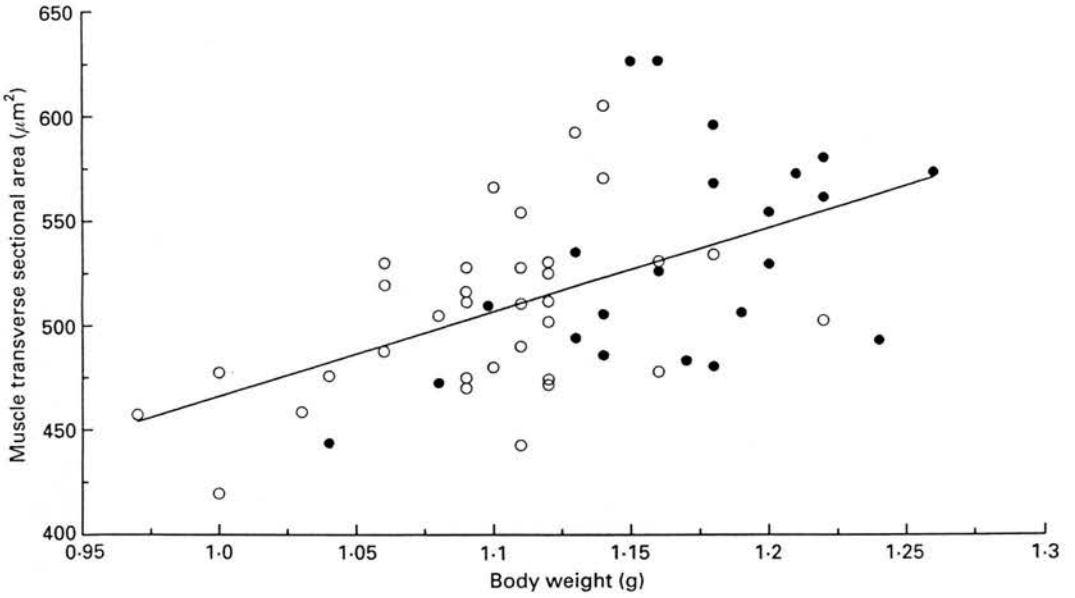

Fig. 5. Relationship between the transverse sectional area of biceps brachii and body weight in the QL and QS mice. The regression equation for the QL and QS data combined were $y = 65.1 + 401x$ ($r^2 = 28.2\%$). ○, QS; ●, QL; —, QL/QS.

Comparisons of bone length at equal body weight in the 2 lines showed bone length to be greater in the QS compared with the QL.

Tibial length was significantly greater in the QL relative to the QS mice at birth ($P \leqslant 0.05$). Analyses of the data show that the percentage increase in the QL relative to the QS was 6.2%. The relationship between the length of the tibia and body weight in the newborn QL and QS mice is shown in Figure 4. A t test of the regression coefficients showed a significant association between tibia length and body weight in both the QL ($P \leqslant 0.001$) and QS ($P \leqslant 0.001$). A comparison between these 2 lines showed a significant difference in the intercepts. As with the humerus, comparisons at equal body weight in the 2 lines indicated that the length of the tibia was greater in the QS mice.

Muscle transverse sectional area in the newborn QL and QS mice

Figure 5 shows the relationship between the transverse sectional area of biceps brachii relative to body weight in the QL and QS mice. A statistical comparison of these 2 lines showed there to be no significant difference between either the slope or intercept. A 2-way analysis of variance showed a significant effect of line ($P \leqslant 0.009$) and littermate size ($P \leqslant 0.006$) on the transverse sectional area of biceps brachii. The mean values showed that muscle transverse sectional area was increased by 13% in the QL relative to the QS mice.

A 2-way analysis of variance showed there to be a significant effect of line on the transverse sectional area of soleus (1% level), although the effect of littermate size was not significant. There was on average a 39% increase in the transverse sectional area of the QL soleus compared to the QS.

Fibre number

Fibre number was evaluated in both muscles in order to determine what proportion of the difference in muscle transverse sectional area between the lines was due to alterations in this parameter. A 2 way analysis of variance was carried out on the data for biceps brachii contained in Table 1 to compare the effect of line (QL/QS) and littermate size. This showed a significant difference between the QL and QS ($P \leqslant 0.025$) mice with respect to fibre number (18% greater in QL) but not between littermates.

In soleus (Table 1) there was a significant effect of both line and littermate size on fibre number ($P \leqslant 0.01$). The overall increase in fibre number within soleus was 17%. Comparisons between the large, average and small littermates of each line showed a 21, 24 and 3% increase, respectively.

Fibre transverse sectional area

Muscle transverse sectional area is mainly a consequence of both fibre number and fibre transverse sectional area. Fibre number was shown in the

Table 1. *Fibre number in biceps brachii and soleus**

	QL biceps	QS biceps	QL soleus	QS soleus
Large littermate	3301±209 (4)	3136±145 (4)	695±19 (3)	572±41 (3)
Average littermate	3302±173 (4)	2646±211 (4)	663±34 (3)	533±26 (3)
Small littermate	3293±252 (4)	2611±341 (4)	508±15 (3)	492±26 (3)
Total mean	3299±3	2798±169	622±58	532±23

* Means±S.E.M. The number of animals on which the mean for each sized littermate is based is shown in brackets.

Table 2. *Fibre transverse sectional area (μm^2) in biceps brachii and soleus**

	QL biceps	QS biceps	QL soleus	QS soleus
Large littermate	67.2±7 (6)	64.8±6 (5)	95.4±6 (3)	58.8±12 (3)
Average littermate	70.8±7 (6)	61.2±6 (5)	91.8±6 (3)	56.7±9 (3)
Small littermate	61.9±6 (6)	58.3±7 (5)	74.8±0.4 (3)	52.7±9 (3)
Total mean	66.6±3	61.5±2	87.3±6.4	56.1±2

* Means±S.E.M. The number of animals on which the mean for each sized littermate is based is shown in brackets.

Table 3. *Nuclear number in transverse sections of biceps brachii**

	Total no. nuclei	Fibre no.	Proportion of fibres with nucleus	No. fibre nuclei	% total nuclear population within fibres
QL mice					
Large littermate (4)	2195±90	3301±206	0.35±0.01	1145±109	52±3.2
Average littermate (4)	2073±187	3165±149	0.28±0.01	895±42	44±3.2
Small littermate (4)	1952±200	3092±216	0.26±0.02	806±9.3	43±4.7
QS mice					
Large littermate (4)	1750±376	3147±205	0.29±0.04	934±160	55±7.2
Average littermate (4)	1824±280	2597±291	0.28±0.01	739±97	40.7±1.5
Small littermate (4)	1478±218	2602±483	0.27±0.02	700±105	47.7±3.7

* Means±S.E.M. The number of animals on which the mean for each sized littermate is based is shown in brackets.

previous section to be increased in the QL relative to the QS mice. Table 2 shows fibre transverse sectional area for biceps brachii within the QL and QS mice. A 2-way analysis of variance showed there to be no significant differences either between the QL and QS ($P = 0.47$) or within the litters ($P = 0.45$). Furthermore, there was no significant correlation between fibre size and body weight in either line (correlation coefficients for the QL 0.28, n = 18 and QS 0.29, n = 15).

Table 2 gives mean fibre transverse sectional area in soleus. A 2-way analysis of variance of these data showed a significant effect of line ($P \leqslant 0.001$) but not littermate size ($P \leqslant 0.37$).

Nuclear number in transverse section

Fibre hyperplasia and hypertrophy are dependent on a supply of nuclei for fusion. The following calculations were therefore performed to determine whether selection had acted to alter the number of nuclei either within or outside of the fibres. Table 3 summarises the data on the total number of nuclei counted in transverse sections of biceps brachii at birth in the QL and QS mice. Column 1 contains the total number of nuclei counted in transverse section, column 2 the fibre number (for the mice used in this particular analysis), column 3 the proportion of fibres showing a nucleus in transverse section, column 4 the total number of fibre nuclei (calculated from the product of columns 2 and 3) and column 5 records the percentage number of the total nuclei contained within the fibres. Analyses of these data were performed using a 2-way analysis of variance to check for the effect of line (QL/QS) or the effect of littermate size within each line. The results of the statistical analyses are shown in Table 4.

Tables 3 and 4 show that the total number of nuclei in transverse section was significantly increased within

Table 4. *Results of a 2-way analysis of variance of the data contained in Table 3*

Comparison	Total no. nuclei	Fibre no.	Proportion of fibres with nucleus	No. fibre nuclei	% total nuclear population within fibres
QL/QS	**	**	n.s.	**	n.s.
Littermates	n.s.	n.s.	n.s.	*	**

* $P \leqslant 0.05$; ** $P \leqslant 0.025$.

the QL compared to the QS mice. Since there was no difference between the lines with respect either to the percentage number of myofibres sectioned with a nucleus (column 3) or to the proportion which this represented of the total nuclear population (column 5), this difference was attributed to the variation in fibre number between the lines. Indeed this conclusion is confirmed by the significant increase seen in the total number of myofibre nuclei of the QL compared with the QS (column 4).

There was no significant difference between littermates with respect to the total number of nuclei (column 1). However, the total number of myofibre nuclei (column 4) together with the proportion of the total nuclear population which this represented (column 5) was significantly reduced in the smaller individuals.

There was no significant effect either of line or of littermate size on the total number of nuclei in transverse sectional area of soleus. Further analyses of the compartmentalisation of the nuclei was not possible due to the method of counting nuclei in this muscle.

DISCUSSION

In agreement with the previous findings of Rucklidge (1982) birth weight was found to differ significantly between the QL and QS. However, birth weight is influenced by environmental as well as by genetic factors (McLaren, 1965) and a negative correlation between mean pup weight and litter size was observed in both the QL and QS mice. Previous work attributes this well known effect to haemodynamic factors within the uterus which effectively determine the level of nutrients delivered to each fetus (McLaren & Mitchie, 1960). Whilst it might be argued that such factors could act differentially in the QL and QS, thereby partly contributing to the differences seen between the lines at birth, the reciprocal transfer of fertilised eggs between high and low body weight Q strain mice has demonstrated that prenatal maternal effects are of limited importance and arise only from variations in litter size (Al-Murrani & Roberts, 1978). It may therefore be concluded that the differences seen in birth weight between the 2 lines, when matched for litter size, are a reflection of an alteration in fetal genotype, brought about as a direct consequence of selection pressure.

These observations do, however, contrast markedly with those previously reported by Penney et al. (1983) who found that body weight was not significantly different between the QL and QS until 10 d postpartum. This discrepancy may be explained in one of two ways. The first and most likely explanation relies upon the fact that both the QL and QS were formed from the combination of several of the replicate lines originally selected by Falconer. Falconer himself noted that selection had led to the fixation of different alleles within each of these replicate lines, although within each size category each has regulated to approximately the same body weight (Falconer, 1973). The combination of several of these replicate lines to form the QL and QS would therefore be expected effectively to increase genetic variation. Since several generations separate the present study and that of Penney et al. (1983) it might be envisaged that the segregation of minor genes, throughout this period, could have led to the observed differences in growth rate.

A second explanation is based upon the failure of Penney et al. (1983) to observe a negative correlation between mean litter weight and litter size in the 2 lines. As this is a well recognised effect in polytocous species such as the mouse (McLaren & Mitchie, 1960; McLaren, 1965; Wahlsten & Bulman-Fleming, 1987), it seems highly unlikely that the QL and QS would be exceptions. Instead, it may be suggested that the number of litters observed for each size category (this information was not reported) was insufficient to permit Penney et al. (1983) to carry out a proper evaluation of the effect of litter size on mean pup weight. The subsequent failure to take an effect of litter size into account when one existed may therefore have led to biased comparisons between the QL and QS.

The lengths of both the humerus and tibia were significantly greater in the QL. This might have been expected since alterations in both the length and diameter of the long bones are known to contribute to genetically determined differences in body weight (Hooper, 1978). However, perhaps a more interesting observation was that the tibia showed a greater percentage change in length than the humerus. This difference between the 2 bones may be related to the pattern of limb development which determines that the rate of growth of the more distal segments (lower limb) exceeds those which are more proximal (upper limb). The difference in the proportional change in length seen between the 2 bones therefore reflects the effect of selection, altering the rate of longitudinal bone growth prenatally.

At comparable body weights, bone length in the QL was less than in the QS. Similar results have previously been reported by Hooper (1978) postnatally, who found that bone length was greater in the QS when the 2 lines were compared at 25 g body weight, whilst the reverse trend was shown if the 2 lines were compared at the same age. These results were taken to imply a relationship between bone length and developmental age as opposed to body weight (Hooper, 1978). Indeed this would seem to apply to the data presented here, since comparisons between the QL and QS at equal birth weight necessarily involves comparisons between the largest QS and the smallest QL littermates. In view of the graded stages of morphological development often evident between fetuses in utero and assuming this is reflected in birth weight, it might very well be suggested that the lowest birth weight QL were developmentally earlier than the larger QS.

Transverse sectional area measurements of biceps brachii and soleus were significantly increased in the newborn QL compared with the QS mice. This increase, together with the changes in bone length discussed above, confirms the previous findings of Hooper et al. (1973) that selection for increased body weight is accompanied by corresponding changes in muscle mass. Muscle weight is determined by 3 parameters: fibre number, size (transverse sectional area and length) and the deposition of extracellular matrix. Since an analysis of fibre density and fibre transverse sectional area in biceps brachii failed to provide any evidence suggesting that the deposition of matrix was altered disproportionately between the lines, the following discussion will be confined to the relative contribution of fibre number and size.

Fibre number in both biceps brachii and soleus was significantly increased in the QL relative to the QS mice. These results are therefore in general agreement with a number of other studies which have shown an association between an increase in fibre number and selection for high body weight (Byrne et al. 1973; Hanrahan et al. 1973; Hooper, 1975; Penney et al. 1983; Stickland & Handel, 1986). However, there are a number of discrepancies with respect to both the pattern and magnitude of response between studies. For example Luff & Goldspink (1967) failed to record any alteration in fibre number within soleus of genetically large and small Q strain mice, whilst both the present study and that carried out by Byrne et al. (1973) reported a significant increase in this variable in soleus. Some diversity in the response of biceps brachii has also been demonstrated, with Byrne et al. (1973) reporting up to a 50% difference in fibre number and Penney et al. (1983) and the present study reporting a 30% and 15% divergence, respectively.

Reasons for the aforementioned variation in response could be severalfold. Previous work has indicated that the magnitude of response may be related to the number of generations of selection pressure which have been applied (Byrne et al. 1973). Hanrahan et al. (1973) and Byrne et al. (1973) both used mice which, whilst originating from the original Q strain, were the result of individual selection programs terminated at 10 and 14 wk, respectively. Others such as Penney et al. (1983) used lines directly descended from those which had been selected by Falconer (1973) for 23 generations. The variation in the number of generations of selection in each case may therefore underlie some of the differences apparent between studies.

A further factor to be taken into consideration is the genetic variation within the individual populations under investigation. Both Penney et al. (1983) and the present study used the QL and QS lines of mice which were originally derived from the combination of several of Falconer's replicate lines (see above discussion). However, the present study reported a 15% divergence in fibre number whilst that by Penney et al. (1983) observed up to a 30% divergence. These differences may only be accounted for by assuming that natural selection, through the number of generations which separate the 2 studies, had led to some alteration in the genetic determinants for fibre number.

The difference of 18% between QL and QS in fibre number of the biceps brachii muscle is similar to the difference found for primary myotube number (S. C. Brown & N. C. Stickland, unpublished observations). This therefore suggests that fibre number differences between the QL and QS in the newborn biceps brachii were due to alterations in primary myotube number

rather than any alteration in the ratio of secondary to primary myotubes. This is in contrast to the situation found by Stickland & Handel (1986) in the pig where genetically determined differences in fibre number were due to alterations in both primary myotube number and the secondary to primary myotube ratio.

Fibre formation and growth are dependent on a supply of nuclei since DNA accretion precedes or closely parallels protein accretion (Moss, 1968*a*, *b*). However, despite the increased growth potential of the QL mice there was no difference in the percentage number of nuclei either within or outside the fibres of biceps brachii at birth. These observations are of considerable importance since they indicate the absence of any alteration in the regulation of fusion within the early muscle, thereby lending support to the hypothesis that selection only acts through the normal cellular processes of growth (Falconer et al. 1978). However, due to the differences in fibre number and fibre length between the 2 lines, the total number of nuclei was increased within the QL biceps brachii compared with the QS. These results are therefore both in agreement with and also explain the biochemical analyses of Penney et al. (1983), who found an increase in the total number of nuclei but no alteration in the protein:DNA ratio between the lines.

Soleus in contrast showed no significant differences between the QL and QS mice with regard to the total number of nuclei in transverse sections of the muscle. This was somewhat surprising since fibre number was significantly increased in the QL compared with the QS, as in biceps brachii. However, since fibre transverse sectional area was also increased in the QL soleus compared with the QS, it might be suggested that this result was due to some change in the protein:DNA ratio incurred as a result of the rapidly diverging growth rates of soleus in the 2 lines. However, to confirm this, further data on the compartmentalisation of nuclei within this muscle would be required.

A comparison of nuclear number in biceps brachii and soleus of differently sized littermates showed no significant difference in either the QL or QS. However, a more detailed analysis in biceps brachii showed that the percentage number of nuclei incorporated into the fibres was significantly reduced in the smaller individuals. These results tend to imply that under conditions of nutritional constraint the fusion of nuclei into the fibre is affected prior to the rate of cell division. On the basis of these observations it seems that whilst genetically and environmentally induced difference in muscle growth may be implemented by separate mechanisms, the basis for both still relies on DNA accretion as was found previously by Moss et al. (1968*a*, *b*) in the chicken.

Selection for divergent muscle weight acts not only on fibre number but also on fibre size (Byrne et al. 1973). Fibre size has 2 main components: fibre transverse sectional area and fibre length. Fibre length in biceps brachii and soleus was estimated by the length of the ossified humerus and tibia respectively. At birth soleus but not biceps brachii showed a significant increase in fibre transverse sectional area, whilst of the 2 bones the tibia showed the greater proportional increase between the QL and QS lines. This would initially seem to lend support to the hypothesis that longitudinal bone growth promotes fibre hypertrophy (Hooper, 1978; Vandenburgh & Karlisch, 1989). However, comparisons between muscles which differ in their fibre type profile may be inherently unreliable in view of probable differences in their pattern of growth (Gibson & Schultz, 1983).

The main aim of the present investigation was to determine the cellular variables associated with the early divergence in size between the QL and QS. Muscle length (as determined by the length of the humerus and tibia) was significantly increased in both the biceps and soleus of the newborn QL compared with the QS. Both muscles showed a significant divergence in fibre number between the lines. Fibre cross-sectional areas were only significantly different between the QL and QS in soleus. This was suggested to be a reflection of the role played by the rate of longitudinal bone growth on fibre hypertrophy since the divergence in the length of the tibia was more marked than that of the humerus. However, further analyses of muscles with different fibre type profiles would be required to confirm this. Detailed analyses of the nuclear content of biceps brachii showed that alterations in fibre number had arisen without any alteration in the proportion of nuclei incorporated into the fibres. On the basis of these results it was concluded that selection pressure had altered fibre number through the normal processes of cellular growth.

REFERENCES

AGUAS AP (1982) The use of osmium tetroxide–potassium ferrocyanide as an extracellular tracer in electron microscopy. *Stain Technology* **57**, 69–73.

AL-MURRANI WK, ROBERTS RC (1978) Maternal effects of body weight in mice selected for large and small size. *Genetical Research Cambridge* **32**, 295–302.

BROWN SC, STICKLAND NC (1993) Satellite cell content in muscles of large and small mice. *Journal of Anatomy* **183**, 91–96.

BYRNE I, HOOPER JC, MCCARTHY JC (1973) Effects of selection for body size on the weight and cellular structure of seven mouse muscles. *Animal Production* **17**, 187–196.

FALCONER DS (1973) Replicated selection for body weight in mice. *Genetical Research Cambridge* **22**, 291–321.

FALCONER DS, GAULD IK, ROBERTS RC (1978) Cell numbers and cell sizes in organs of mice selected for large and small body size. *Genetical Research Cambridge* **31**, 287–301.

GIBSON MC, SCHULTZ E (1983) Age related differences in absolute numbers of skeletal muscle satellite cells. *Muscle and Nerve* **6**, 574–580.

HANDEL SE, STICKLAND NC (1984) Muscle cellularity and its relationship with birth weight and growth. *Journal of Anatomy* **139**, 726.

HANRAHAN JP, HOOPER AC, MCCARTHY JC (1973) Effects of divergent selection for body weight on fibre number and diameter in two mouse muscles. *Animal Production* **16**, 7–16.

HOOPER AC (1975) The relative contribution of the components of muscle growth. *Journal of Anatomy* **120**, 414.

HOOPER ACB (1978) Muscles and bones of large and small mice compared at equal body weights. *Journal of Anatomy* **127**, 117–123.

HOOPER AC, BYRNE I, MCCARTHY JC (1973) The effects of selection for body weight on muscle structure in mice. *Journal of Anatomy* **115**, 146.

LUFF AR, GOLDSPINK G (1967) Large and small muscles. *Life Sciences* **6**, 1821–1826.

MCLAREN A (1965) Genetic and environmental effects on foetal and placental growth in mice. *Journal of Reproduction and Fertility* **9**, 79–98.

MCLAREN A, MITCHIE D (1960) Control of prenatal growth in mammals. *Nature* **187**, 363–365.

MILLER JB, STOCKDALE FE (1986) Developmental regulation of the multiple myogenic cell lineages of the avian embryo. *Journal of Cell Biology* **103**, 2197–2208.

MOSS FP (1968*a*) The relationship between the dimensions of the fibres and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. *American Journal of Anatomy* **122**, 535–564.

MOSS FP (1968*b*) The relationship between the dimensions of the fibres and the number of nuclei during restricted growth, degrowth and compensatory growth of skeletal muscle. *American Journal of Anatomy* **122**, 565.

PENNEY RK, PRENTIS PF, MARSHALL PA, GOLDSPINK G (1983) Differentiation of muscle and the determination of ultimate tissue size. *Cell and Tissue Research* **228**, 375–388.

REYNOLDS ES (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

RUCKLIDGE GJ (1981) Differences in body compositions, growth and food intakes between mice which have been selected for a small and large body size. *British Journal of Nutrition* **46**, 441–450.

RUCKLIDGE GJ (1982). Differences in body compositions, growth and food intakes between mice which have been selected for small or large body size. Effect of plane of neonatal nutrition. *British Journal of Nutrition* **48**, 341–352.

STICKLAND NC, HANDEL SE (1986) The numbers and types of muscle fibres in large and small breeds of pigs. *Journal of Anatomy* **147**, 181–189.

VANDENBURGH HH, KARLISCH P (1989) Longitudinal growth of skeletal myotubes in vitro in a new horizontal mechanical cell stimulator. *In Vitro Cellular and Developmental Biology* **25**, 607–616.

WAHLSTEN D, BULMAN-FLEMING B (1987) The magnitudes of litter size and sex effects on brain growth of BALB/c mice. *Growth* **51**, 240–248.

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The Influence of Male-Specific Genes on Female Muscle Fiber Types: Studies on the Sex-Reversed (*Sxr*) Mouse

NEIL C. STICKLAND AND PETER J. O'SHAUGHNESSY
Department of Veterinary Basic Sciences, The Royal Veterinary College, London NW1 OTU (N.C.S.), and Department of Veterinary Physiology, University of Glasgow Veterinary School, Glasgow G61 1QH (P.J.O.), United Kingdom

ABSTRACT Previous experiments on the effects of either male castration or injection of androgens have concluded that levels of androgens are responsible for different muscle fiber type proportions between the sexes. However, these conclusions are based on invasive techniques which may involve secondary factors. The sex-reversed (*Sxr*) mouse is genetically female (X/X) but phenotypically male due to the presence of part of the short-arm of the Y chromosome containing the testis determining gene (*Tdy*). Serum testosterone in this mouse is in the low normal range and therefore provides a model for investigating the possible control of muscle fiber types by male specific genetic factors.

Ten males, ten females, and ten *Sxr* mice of approximately 60 days of age were used, together with ten males weight-matched to the females. The animals were killed and biceps brachii and soleus muscles removed and prepared for routine muscle histochemistry. Body and muscle weights were similar in the males and *Sxr* mice and significantly greater than in the females and the weight-matched males. Muscle fiber sizes in biceps brachii reflected the differences in muscle weights and there were no significant differences in fiber type proportions for this muscle. In the soleus muscle, the percentage of slow, oxidative (SO) fibers was higher in the female mice than in any other group. Furthermore, although the fast, oxidative, glycolytic (FOG) fibers were larger in the heavier animals, SO fibers were largest in the female mice.

The results indicate that, although the size of FOG and fast, glycolytic (FG) fibers may be a result of work-load hypertrophy related to body weight, the proportion and size of SO fibers in the soleus is directly influenced by genetic factors derived from the Y-chromosome, probably *Tdy*.

It is well known in many species that, at the same age, adult males are heavier than adult females. These differences have been shown in some studies to be reflected in a difference in muscle weights with certain muscles more affected than others. Investigations into the fiber type composition of these more sexually dimorphic muscles have also shown differences between the sexes (e.g., levator ani of the rat; Fishman and Breedlove, '80). These sex differences have generally been attributed to the presence of testicular-derived androgens in males although castration experiments have been conflicting. The castrated mouse has been shown to exhibit fiber type proportions in its soleus muscle similar to those in the female, which in both are different from the intact male (Vaughan et al., '74). In another study, however, castration had no affect on fiber type proportions in the extensor digitorum longus (Jiang and Klueber, '89). Injection of androgens has been shown to affect fiber type proportions in the highly androgen-sensitive muscles (Kelly et al., '85). There are some potential problems, however, with using castration experiments in this way. Androgen production by the testis begins soon after testicular differentiation and androgen levels, in many species, reach a peak around birth (Gondos, '80). In animals which are subsequently castrated, even soon after birth, there will have been exposure to significant levels of androgen during development. In addition, the effects of castration are more widespread than simply removal of testicular androgen. Circulating levels of several hormones will be altered by this procedure including

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Address reprint requests to Prof. N.C. Stickland, Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 OTU, U.K.

thyrotropin and growth hormone (Christianson et al.., '81; Jansson and Frohman, '87). Similarly in females treated with androgens the animals will not have been exposed to male levels of androgen during fetal development. An alternative approach to the study of sex influences on muscle development is use of the sex-reversed (*Sxr*) mouse. The *Sxr* mutation causes genetic (X/X) females to develop testes due to the presence of part of the short arm of the Y-chromosome carrying the testis determining gene (*Tdy*) attached to the distal end of one of the X-chromosomes (Evans et al., '82; McLaren et al., '88). We therefore have a model in this mouse for investigating the possible control of muscle fiber types by genetic factors specific to the short-arm of the Y-chromosome.

The purpose of this investigation was first to determine what differences there are between the muscles of male and female mice and second, to investigate whether these differences are due to genes associated with the short arm of the Y-chromosome, including *Tdy*. A sex effect on muscles may be mediated through differences in body weight, e.g., body weight has been shown to affect slow fiber content in muscles (Handel and Stickland, '87). It was therefore important to control for this also in this study. The muscles chosen for this study were the biceps brachii, which is predominantly a fast muscle in the mouse, and the soleus muscle, which has a relatively high slow fiber content being predominantly a postural muscle. Neither muscle is of known high androgen-sensitivity. The characterization of both fast and slow muscles as influenced by genetic sex, weight, and androgen levels were investigated.

MATERIALS AND METHODS

Ten males, ten females, and ten *Sxr* mice from the same background strain and all of approximately 60 days of age were used for this study. A fourth group of male mice, weight-matched to the females, was also used. These mice were approximately 35 days of age. The animals were killed by decapitation and the blood collected for hormone analysis (O'Shaughnessy and Sheffield, '90). The testes and epididymides were removed and weighed. The muscles used for this study were the biceps brachii (a predominantly fast muscle) and the soleus (a mixed muscle with a relatively high slow fiber content). These two muscles were removed, weighed, and then frozen in dichlorodifluoromethane (Arcton 12, ICI Ltd, Macclesfield, UK) cooled to its melting point of –158°C in liquid nitrogen. Transverse sections (10 μm) of the muscles were obtained in the midbelly region on a cryostat. Histochemical tests were employed on the sections to determine the activity of the following enzymes: Succinic dehydrogenase (SDHase) (Nachlas et al., '57), Glycogen phosphorylase (GPase) (Takeuchi, '56), alkali-stable Adenosine triphosphatase (b-ATPase), and acid-stable ATPase (a-ATPase) (Guth and Samaha, '70). The use of serial sections for these tests enabled muscle fibers to be classified as slow, oxidative (SO), fast, glycolytic (FG), or fast oxidative, glycolytic (FOG). For each muscle the proportion of each fiber type present and the mean cross-sectional area (based on 100 fibers) of each type was estimated using a Seescan Image Analysis System (Seescan plc, Cambridge, UK). Differences between the four groups of mice were assessed by analysis of variance followed by a Newman-Keul test.

RESULTS

The males and *Sxr* mice were similar in body weight and were significantly heavier than the females and the lighter (weight-matched) males (Fig. 1). The differences in body weight were reflected in the differences in weight of both biceps brachii and soleus muscles (Table 1). There was, in fact, no difference between groups of mice when the muscle weights were expressed as a percentage of body weight. Serum testosterone levels were much higher in the males than the females. Levels in the *Sxr* mice were not as high as in the normal males.

As expected the total number of muscle fibers in the soleus muscle was not different between

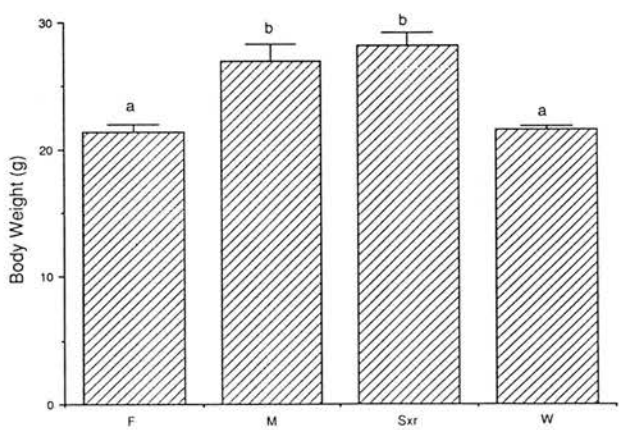

Fig. 1. Mean body weights for females (F), males (M), *Sxr*, and weight-matched (W) males. In each group, n = 10. Error bars are standard errors of the means. Groups showing the same letter are not significantly different by the Newman-Keul test.

TABLE 1. Serum testosterone levels and muscle weights

	Female	Male	*Sxr*	Weight-matched male
Serum testosterone (fmol/ml)	ND (< 250)	4,841	1,190	2,200
Biceps brachii weight (mg)	14.4	19.9	20.3	14.3
% of body weight	0.068	0.069	0.070	0.066
Soleus weight (mg)	5.02	6.73	6.23	4.61
% of body weight	0.022	0.023	0.021	0.021

ND, not detectable.

the groups (Fig. 2). This parameter was not measured in the biceps brachii muscle owing to the much larger size of this muscle. The soleus muscles contained SO and FOG fibers only. The percentage of SO fibers was significantly higher in the soleus muscle of the female mice than in any of the other three groups (Fig. 3). Furthermore, the mean cross-sectional area of the soleus SO fibers was also greater in the female mice (Fig. 4a). The FOG fibers in the soleus muscles tended, however, to be larger in the bigger males and in the *Sxr* mice (Fig. 4b).

As for the biceps brachii muscle, the differences in body weights were reflected in the sizes of both the FOG and FG fibers (Fig. 5) in that both fiber types were larger in the larger males and *Sxr* mice than in the females and lighter (weight-matched) males. FOG and FG fibers were the only fiber types in the biceps brachii muscles except in the normal females. In four of ten of the females there was a very small proportion of SO fibers (less than 1%) with the highest proportion in the youngest and lightest female.

DISCUSSION

Serum androgen levels in *Sxr* mice were significantly lower than in normal adult males as described previously (Daley and Younglai, '78) and this may be related to alterations in testicular blood flow (O'Shaughnessy et al., '91). The results show, however, that the levels of androgens in the *Sxr* mice were sufficient to increase their body weights to those of normal males. In all the muscle parameters tested there was no difference between normal adult males and *Sxr* mutants although both differed significantly from adult females as discussed below. This would confirm that differences in muscle function between males and females are related to genetic factors associated with the short arm of the Y-chromosome. The number of genes present on this portion of the chromosome in unknown but includes *Tdy* and it is likely that androgens derived from the testes of *Sxr* mice are responsible for maintaining the male phenotype of the muscles tested.

Not surprisingly the differences in body weights were reflected in differences in muscle weights and

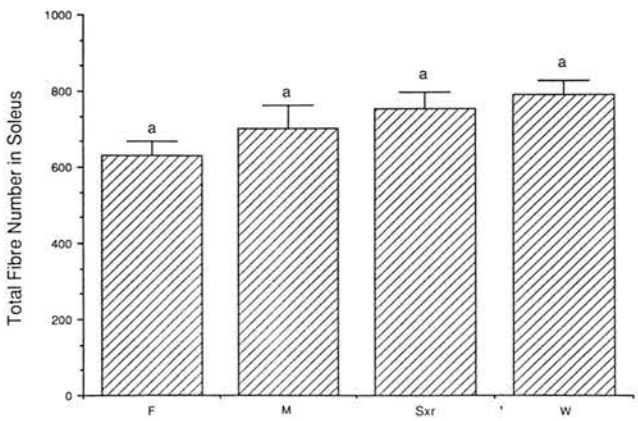

Fig. 2. Total muscle fiber number in the soleus muscles of females (F), males (M), *Sxr*, and weight-matched (W) males. In each group, n = 10. Error bars are standard errors of the means. Groups showing the same letter are not significantly different by the Newman-Keul test.

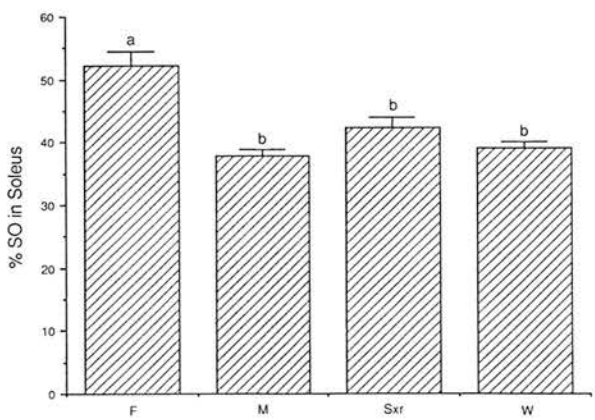

Fig. 3. Percentage of SO fibers in the soleus muscle of females (F), males (M), *Sxr*, and weight-matched (W) males. In each group, n = 10. Error bars are standard errors of the means. Groups showing the same letter are not significantly different by the Newman-Keul test.

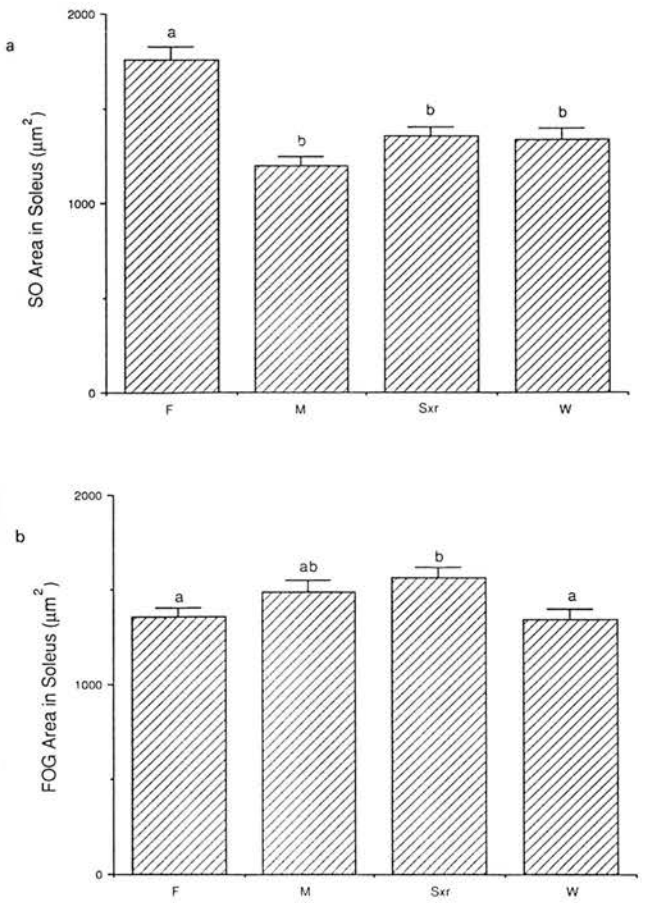

Fig. 4. Mean fiber areas for (**a**) SO fibers and (**b**) FOG fibers in the soleus muscles of females (F), males (M), *Sxr*, and weight-matched (W) males. In each group, n = 10. Error bars are standard errors of the means. Groups showing the same letter are not significantly different by the Newman-Keul test.

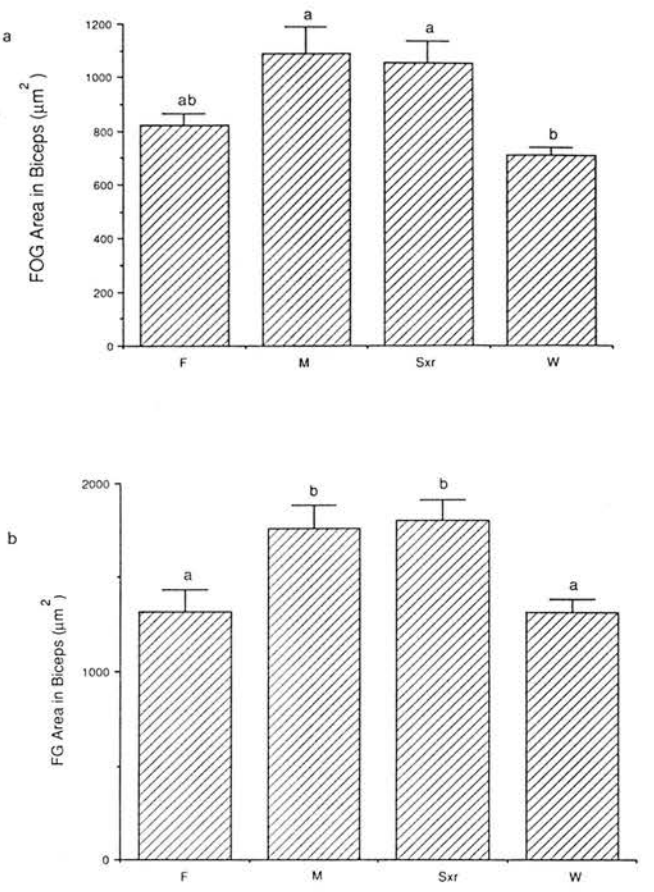

Fig. 5. Mean fiber areas for (**a**) FOG fibers and (**b**) FG fibers in the biceps brachii muscle of females (F), males (M), *Sxr*, and weight-matched (W) males. In each group, n = 10. Error bars are standard errors of the means. Groups sharing the same letter are not significantly different by the Newman Keul test.

in the sizes of their constituent fibers (apart from the SO fibers in the soleus). There was no evidence in this investigation for a difference in relative muscle weights (expressed as a percentage of body weight) between the groups of mice. Both the biceps brachii (a fast muscle) and the soleus (a postural, slower muscle) maintained the same percentage of body weight in each group. There is evidence, however, from other work that some muscles in the mouse (Rowe, '68), rat (Fishman and Breedlove, '80), and guinea pig (Kelly et al., '85) may be directly androgen-sensitive. For the biceps brachii and soleus muscles in this investigation the differences can be attributed to differences in work-load wrought by body weight differences.

A parameter that was not related to body weight differences was the proportion of SO fibers in the soleus muscle. The proportion of these SO fibers in the soleus was significantly higher in the female mice than in the other three groups. The incidence of SO fibers in the biceps brachii of female mice alone corroborates the finding in the soleus. The fact that this is not a weight-related result is highlighted by the lower proportion of SO fibers in the lighter males (matched in weight to the females). Furthermore any affect which has noted a relationship between SO fiber content and bodyweight has shown that the proportion of SO fibers increases with bodyweight (Handel and Stickland, '87). In this situation the lighter females have more SO fibers in the soleus than the heavier males. The results suggest that androgens may be responsible for a transition from slow to fast contractile proteins within the soleus muscle. Androgens have been shown to affect fiber type

transitions in other studies. Testosterone administration will stimulate transition from FOG to FG fibers in the temporalis muscle of the guinea pig (Kelly et al., '85) and myosin heavy chain isoenzymes in ventricular muscle are also affected by testosterone levels in rats (Schever et al., '87).

The mechanism of action of androgens on muscle is unclear. A direct effect would be mediated through the androgen receptor and one might speculate that muscle sensitivity might be related to receptor density. It has also been suggested that the action of testosterone on muscle might be mediated through growth hormone (Kawai et al., 1982). There is also likely to be a relationship with thyroxine, which is known to be sex-dependent and also to have a profound effect on expression of contractile proteins in muscle tissue (Christianson et al., 1981).

A further interesting finding from this investigation is the large size of the SO fibers in the soleus muscle of female mice compared to the other three groups of mice. This again is not a weight-related parameter. If it is suggested that androgens are responsible for the increase in fast fibers it is difficult to explain the stimulus which increases the size of the SO fibers (which are already greater in number in the females).

In conclusion, it would appear that, whereas the size of FOG and FG fibers may be a result of workload hypertrophy related to body weight, the proportion and size of SO fibers in the soleus, and possibly other muscles, is directly influenced by genetic factors, probably acting through formation of the testes.

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LITERATURE CITED

Christianson, D., E. Roti, A.G. Vagenakis, and L.E. Braverman (1981) The sex-related difference in serum thyrotropin concentration is androgen mediated. Endocrinology, *108:*529–535.

Daley, J.D., and E.V. Younglai (1978) Steroid metabolism in testicular tissue of genetic mutant mice. J. Steroid Biochem., *9:*41–45.

Evans, E.P., M.D. Burtenshaw, and B.M. Cattanach (1982) Meiotic crossing-over between the X and Y chromosomes of male mice carrying the sex-reversing (*Sxr*) factor. Nature, *300:*443–445.

Fishman, R.B., and S.M. Breedlove (1980) Neonatal androgen maintains sexually dimorphic muscles in the absence of innervation. Muscle Nerve, *11:*553–560.

Gondos, B. (1980) Development and differentiation of the testis and male reproductive tract. In: Testicular Development, Structure and Function. A. Steinberger and E. Steinberger, eds. Raven Press, New York. pp. 3–20.

Guth, L., and F.J. Samaha (1970) Research note: Procedure for the histochemical demonstration of actomyosin ATPase. Exp. Neurol., *28:*365–367.

Handel, S.E., and N.C. Stickland (1987) The growth and differentiation of porcine skeletal muscle fiber types and the influence of birthweight. J. Anat., *152:*107–119.

Jansson, J.O., and L.A. Frohman (1987) Differential effects of neonatal and adult androgen exposure on the growth hormone secretory pattern in male rats. Endocrinology, *120:*1551–1557.

Jiang, B., and K.M. Klueber (1989) Structural and functional analysis of murine skeletal muscle after castration. Muscle Nerve, *12:*67–77.

Kawai, K., E. Ogata, K. Tukano, H. Hizuka, K. Yamashitaka, and K. Shizume (1982) Effects of testosterone and estradiol on serum SmA and growth rate of rats. Endocrinol. Jpn., *29:*435–442.

Kelly, A., G. Lyons, B. Gambki, and N. Rubinstein (1985) Influences of testosterone on contractile proteins of the guinea pig temporalis muscle. Adv. Exp. Med. Biol., *182:*155–168.

McLaren, A., E. Simpson, J.T. Epplen, R. Studer, P. Koopman, E.V. Evans, and P.S. Burgoyne (1988) Location of the genes controlling H-Y antigen expression and testis determination on the mouse Y chromosome. P.N.A.S. U.S.A., *85:*6442–6445.

Nachlas, M.M., K.C. Tsou, E. De Souza, C.S. Cheng, and A.M. Seligman (1957) Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem., *5:*420–436.

O'Shaughnessy, P.J., and J.W. Sheffield (1990) Effect of testosterone on testicular steroidogenesis in hypogonadol (*hpg*) mouse. J. Steroid Biochem., *35:*729–734.

O'Shaughnessy, P.J., D.H. Abbott, A.J. Leight, and B.M. Cattanach (1991) Testicular steroidogenesis in X/X sex-reversed mice. Int. J. Androl., *14:*140–148.

Rowe, R.W.D. (1968) Effect of castration on muscle growth in the mouse. J. Expt. Zool., *168:*59–64.

Schever, V., A. Malhotra, T.F. Schiable, and J. Capasso (1987) Effects of gonadectomy and hormonal replacement on rat hearts. Circ. Res., *61:*12–19.

Takeuchi, T. (1956) Histochemical demonstration of phosphorylase. J. Histochem. Cytochem., *4:*84.

Vaughan, H.S., Aziz-Ullah, G. Goldspink, and N.W. Nowell (1974) Sex and stock differences in the histochemical myofibrillar adenosine triphosphatase reaction in the soleus muscle of the mouse. J. Histochem. Cytochem., *22:*155–159.

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N.C. Stickland
A.R. Crook
C.M. Sutton

Department of Veterinary Basic Sciences,
The Royal Veterinary College, London, UK

Effects of Pituitary Dwarfism in the Mouse on Fast and Slow Skeletal Muscles

Key Words
Skeletal muscles
Fast
Slow
Pituitary
Dwarf mouse

Abstract
The Snell dwarf mouse exhibits impaired growth of the anterior pituitary resulting in reduced levels of growth hormone and thyroid stimulating hormone. Ten dwarf mice and 10 phenotypically normal littermates were killed at 33 days of age. M. biceps brachii (a predominantly fast muscle) and m. soleus (a relatively slow muscle) were removed from each animal and complete frozen transverse sections obtained. Serial sections were reacted for various enzyme activities in order to identify muscle fibre types. There was no difference in the total number of muscle fibres in m. biceps brachii but a small difference in m. soleus between normal and dwarf mice. There were marked differences in the size of all fibre types between normal and dwarf mice with the largest differences in m. soleus. The percentage of slow oxidative fibres was similar (about 32%) in both groups of mice for m. soleus but there was a marked difference for this fibre type in m. biceps brachii being about 1.5% in normal mice and 8.0% in dwarf mice. This may be related to a difference in levels of thyroid hormone. Nuclear density was very significantly greater in dwarf muscles although total nuclear numbers were less than in normal muscles. These differences are most likely due to growth hormone levels. Differences in nuclear content were much greater in m. soleus than in m. biceps brachii.

Introduction

The Snell dwarf mouse (dw) exhibits impaired anterior pituitary growth which has a consequent effect on growth hormone and thyroid-stimulating hormone [van Buul-Offers, 1983]. This results in reduced growth in the mutants. Although the endocrinology of the Snell dwarf mouse has been studied in some detail, there is a lack of comprehensive data on the characterisation of the muscles in these animals. Experimental manipulation of growth levels [Cheek and Hill, 1970] and thyroid hormone levels [D'Albis et al., 1987] have indicated the importance of these hormones in normal muscle growth. This has been highlighted more recently by inserting growth hormone gene into mice [Palmiter et al., 1982] which grew bigger, and pigs [Pursel et al., 1990] which grew faster and more efficiently. The muscles of such animals, however, have not been studied in detail. The Snell dwarf mouse provides an opportunity to study the effect of genetically altered levels of anterior pituitary hormones on muscle growth and to compare the results with those utilising more experimental techniques to study the effect of these hormones. The hypothesis to be tested is that differences in the muscles between dwarf and normal mice can be attributed to different levels of growth hormone and thyroid hormone as revealed by previous experimental studies.

Abbreviations used in this paper:
ATPase = Adenosine triphosphatase; CSA = cross-sectional area; FG = fast glycolytic; FOG = fast oxidative glycolytic; SO = slow oxidative.

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Prof. N.C. Stickland
Department of Veterinary Basic Sciences
The Royal Veterinary College
Royal College Street
London NW1 OTU (UK)

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Table 1. Gross parameters (means ± SE) for dwarf mice and normal phenotype at 33 days of age

Parameter	Dwarf	Normal	Ratio normal:dwarf
n	10	10	
Body weight, g	6.24±0.35	17.98±0.51	2.9
Biceps brachii weight, mg	3.22±0.34	10.69±0.43	3.3
Soleus weight, mg	0.84±0.11	3.90±0.17	4.6
Biceps brachii length, mm	3.69±0.20	5.23±0.16	1.4
Soleus length, mm	3.33±0.08	5.35±0.27	1.6
Biceps brachii CSA, mm^2	1.36±0.13	2.53±0.14	1.9
Soleus CSA, mm^2	0.39±0.04	1.18±0.09	3.0

All differences significant at $p < 0.001$. CSA = Cross-sectional area.

Materials and Methods

Ten Snell dwarf mice and 10 phenotypically normal littermates were sacrificed at 33 days of age. Various gross measurements (included in table 1) were noted. M. biceps brachii (a predominantly fast muscle) and m. soleus (a relatively slow muscle) were removed from each animal and frozen immediately in liquid dichlorodifluoromethane (Arcton 12, ICI) cooled to −158 °C in liquid nitrogen. Complete 10-µm transverse sections were cut on a cryostat through the midbelly region of each muscle. Serial sections from each muscle were reacted for the following enzymes: alkali (pH 10.2) and acid-stable (pH 4.6) myosin ATPase (based on Guth and Samaha [1970]), succinate dehydrogenase [Nachlas et al., 1957] and glycogen phosphorylase [Takeuchi, 1956]. Using these reactions muscle fibres were classified as slow oxidative (SO), fast oxidative glycolytic (FOG) or fast glycolytic (FG). M. biceps brachii was found to contain all three fibre types whereas m. soleus contained only SO and FOG fibres. Using a projection microscope, under low magnification, the complete cross-sectional area (CSA) of each muscle was measured and the total numbers of each fibre type making up each muscle were counted. Under higher magnification the cross-sectional areas of 100 fibres of each type for each muscle were measured using an Image analysis system (Seescan plc, Cambridge). Sections stained with haematoxylin and eosin were used to count the number of nuclei in transverse sections for each muscle. The procedure given by Abercrombie [1946] was used to adjust these figures for section thickness. The number of nuclei per mm^3 (N^1) was calculated by the formula:

$$\frac{N}{A \cdot T},$$

where N = adjusted number per section, A = muscle cross-sectional area and T = section thickness. A figure for total number of nuclei per muscle was obtained from the formula:

$$N^1 \cdot A \cdot L,$$

where L = length of the muscle. These figures made no allowance for tapering ends of muscle and so would be overestimates but as relative values were of interest in this investigation this was not considered a problem. Differences between normal and dwarf animals for various parameters were assessed by Student's t test.

Results

The gross parameter measurements are given in table 1. It can be seen that for all gross muscle values the differences between normal and dwarf mice were greater for the soleus muscle than the biceps brachii muscle.

The cellular parameters of the muscles studied are shown in figures 1–5. Figure 1 shows the results of alkali-stable ATPase reactions on the muscle sections. It must be stressed that the identification of fibres was, however, based on analysis of all three of the histochemical methods mentioned above. In biceps brachii sections (fig. 1) the darkest fibres were FOG, intermediate were FG and the lightest were SO. The same differential staining was also seen by Goldspink and Ward [1979] in murine biceps brachii. In the soleus sections, although some differential staining of fast fibres was evident (fig. 1), all dark and intermediate fibres were FOG according to their reactions for succinate dehydrogenase and glycogen phosphorylase.

There was no statistically significant difference between normal and dwarf mice for muscle fibre number in m. biceps brachii, although there did appear to be a small significant difference (17%) for m. soleus (fig. 2). Differences in muscle fibre CSA were large and differences were greater in m. soleus than in m. biceps brachii (fig. 3). A very significant difference was found in the percentage of SO fibres in biceps brachii and a small difference in m. soleus (fig. 4). Nuclear concentration in the dwarf muscles was approximately 1.6 and 2.0 times greater than in the normal for biceps and soleus, respectively (fig. 5a). Total nuclei number in the dwarf muscles was approximately 60 and 40% of the normal values for biceps and soleus, respectively (fig. 5b).

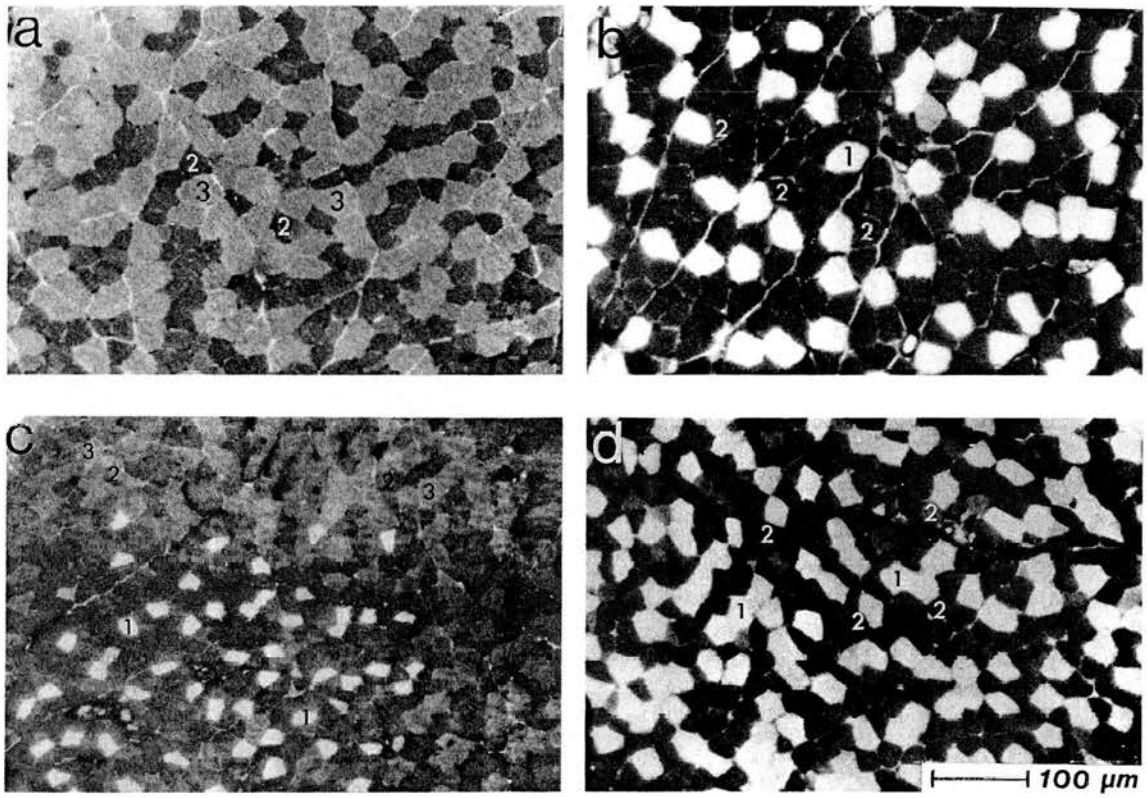

Fig. 1. Transverse sections reacted for alkali-stable myosin ATPase from (**a**) control m. biceps brachii; (**b**) control m. soleus; (**c**) dwarf m. biceps brachii, and (**d**) dwarf m. soleus. Fibre typing is based on three histochemical reactions. Muscle fibres are labelled 1 (SO, slow oxidative); 2 (FOG, fast oxidative glycolytic); 3 (FG, fast glycolytic).

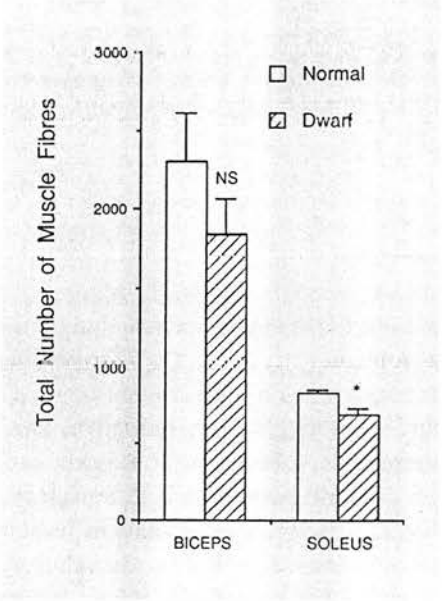

Fig. 2. Total number of muscle fibres in m. biceps brachii and m. soleus for normal and dwarf mice. Error bars are SEMs. Differences between normal and dwarf: NS = not significant; *p < 0.015.

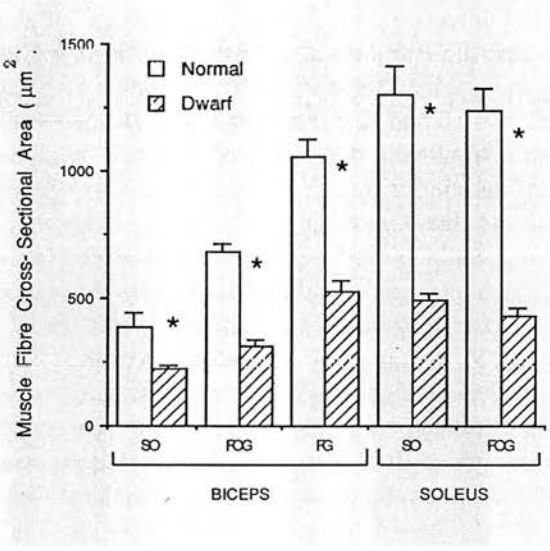

Fig. 3. Mean muscle fibre cross-sectional areas for each fibre type in m. biceps brachii and m. soleus for normal and dwarf mice. Error bars are standard errors of the means. SO = Slow oxidative; FOG = fast oxidative glycolytic; FG = fast glycolytic. For all fibre types differences between normal and dwarf are *p < 0.001.

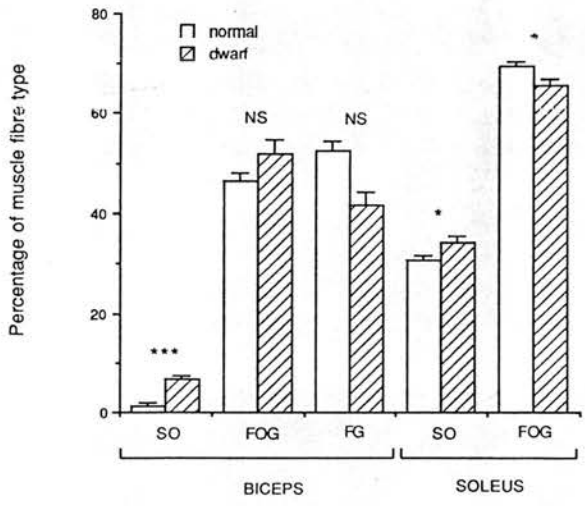

Fig. 4. Percentage of slow oxidative (SO), fast oxidative glycolytic (FOG) and fast glycolytic (FG) fibres in m. biceps brachii and m. soleus for normal and dwarf mice. Error bars are SEMs. Differences between normal and dwarf: NS = not significant; ***$p < 0.001$; *$p < 0.05$.

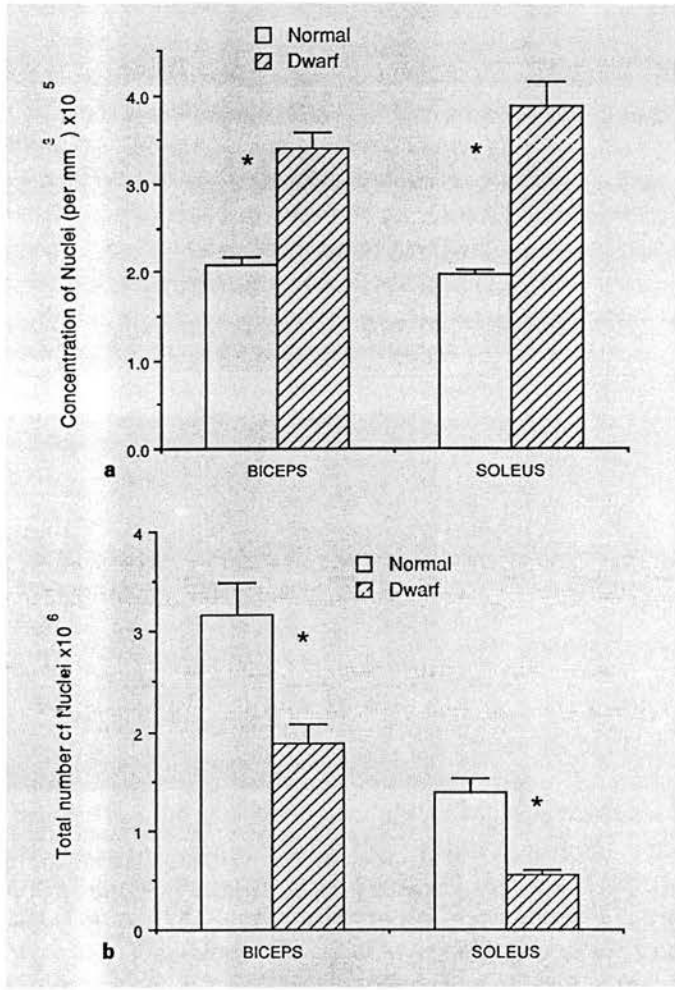

Fig. 5. Concentration (**a**) and total number (**b**) of nuclei in m. biceps brachii and m. soleus for normal and dwarf mice. Error bars are SEMs. Differences between normal and dwarf, for each muscle, are *$p < 0.001$.

Discussion

Impaired anterior pituitary growth has an obvious effect on the growth of skeletal muscles. In the Snell dwarf mouse structural defects in the growth of the anterior pituitary are not evident until 5 days after birth [Francis, 1944]. In the mouse there is no new muscle fibre formation after the time of birth [Ontell and Kozeka, 1984]. It is, therefore, surprising that a significant difference, albeit small, was found in the total number of muscle fibres in m. soleus between the normal and dwarf mice (fig. 2). As it is unlikely that this difference was achieved prenatally a possible explanation is that the reduced growth stimuli to the muscles caused the smaller fibres to gradually degenerate in the dwarf mice. However, Slabaugh et al. [1981] concluded that growth hormone deficiencies (in contrast to structural defects in the anterior pituitary) could be detected at the time of birth in the Snell mouse. It is also claimed by some workers that growth hormone may be important in prenatal growth [Kim et al., 1993]. There is, therefore, a possibility that the dwarf mouse exhibits reduced prenatal muscle fibre hyperplasia. A growth study from fetal to postnatal ages would be required to clarify this point.

The difference in size of all constituent muscle fibre types in both muscles is very evident between normal and dwarf animals (fig. 3). It is clear that this difference is greater in the soleus muscle than in the biceps brachii. This is probably related to the fact that the soleus is a postural muscle; it has a high content of slow fibres (about a third) and all of its fibres are oxidative. The biceps brachii is predominantly a fast muscle. It is known that postural muscles (or postural parts of mixed muscles) grow relatively more than fast muscles as animals increase in body weight [Handel and Stickland, 1987]. The relatively larger difference in muscle fibre size of the soleus compared to the biceps is, therefore, a function of the difference in body weight.

It is known from studies of the postnatal growth of fast muscles, such as m. biceps brachii of the hamster [Gold-

spink and Ward, 1979], that the small proportion of SO fibres decreases with growth. This is in contrast to postural muscles in which the proportion of SO fibres increases with growth [Kugelberg, 1976; Handel and Stickland, 1987]. The conversion of slow to fast fibres appears to be under the control of thyroid hormone [D'Albis et al., 1987]. The fact that the Snell dwarf mouse has low thyroid gland activity may, therefore, be the reason for the higher proportion of SO fibres in the dwarf biceps compared to the normal (fig. 4).

The higher concentration of nuclei, but lower total number of nuclei in the muscles of dwarf compared to normal mice is a reflection of lower protein synthesis and reduced nuclear division, respectively. Both of these activities in muscle can be related to levels of growth hormone [Cheek and Hill, 1970]. Again, the effect is more marked in the soleus than in the biceps for the reasons outlined above concerning the relatively greater stimulus to slow muscle as animals increase in body size. Another contributory factor in the dwarf mouse may be its more sluggish behaviour associated with its hypothyroidism [Boettiger, 1941] which would invoke less functional demand on its fast muscles compared to normal mice.

Taken as a whole the differences found between genetically dwarf and normal muscles are consistent with the results from other studies which used experimental manipulation of growth hormone and thyroid hormone levels. However, it is clear that more experiments are required to address specific roles of these hormones acting alone versus in concert.

Acknowledgement

The authors are grateful to Dr. A. Holder for providing the animals used in this investigation.

References

Abercrombie, M. (1946) Estimation of nuclear population from microtome sections. Anat Rec *94:* 239–247.

Boettiger, E.G. (1941) The effect of thyroxin on growth, oxygen consumption and body composition of hereditary dwarf mice. Endocrinology *28:* 785–792.

Buul-Offers, S. van (1983) Hormonal and other inherited growth disturbances in mice with special reference to the Snell dwarf mouse. A review. Acta Endocrinol *103* suppl: 258.

Cheek, D.B., D.E. Hill (1970) Muscle and liver cell growth: Role of hormones and nutritional factors. Fed Proc *29:* 1503–1509.

D'Albis, A., M. Lenfant-Guydt, C. Janmot, C. Chanoine, J. Weinham, C.L. Gallien (1987) Regulation by thyroid hormones of terminal differentiation in skeletal dorsal muscle. 1. Neonate mouse. Dev Biol *123:* 25–32.

Francis, T. (1944) Studies on hereditary dwarfism in mice. VI. Anatomy, histology and development of the pituitary at hereditary anterior pituitary dwarfism in mice. Acta Pathol Microbiol Scand *21:* 928–944.

Goldspink, G., P.S. Ward (1979) Changes in rodent muscle fibre types during post-natal growth, undernutrition and exercise. J Physiol *296:* 453–469.

Guth, L., F.J. Samaha (1970) Research note: Procedure for the histochemical demonstration of actomyosin ATPase. Exp Neurol *28:* 365–367.

Handel, S.E., N.C. Stickland (1987) The growth and differentiation of porcine skeletal muscle fibre types and the influence of birthweight. J Anat *152:* 101–119.

Kim, J.D., K. Näntö-Salonen, J.R. Szczpankiewicz, R.G. Rosenfeld, G.F. Glasscock (1993) Evidence for pituitary regulation of somatic growth, IGF-1 and -2, and their binding proteins in the fetal rat. Pediat Res *33:* 144–151.

Kugelberg, E. (1976) Adaptive transformation of rat soleus motor units during growth. Histochemistry and contraction speed. J Neurol Sci *27:* 269–289.

Nachlas, M.M., K. Tsouk, F. DeSousa, C. Cheng, M. Seligman (1957) Cytochemical demonstration of succinic dehydrogenase by the use of a new *p*-nitrophenol substituted ditetrazole. J Histochem Cytochem *5:* 420–436.

Ontell, M., K. Kozeka (1984) Organogenesis of the mouse extensor digitorum longus muscle: A quantitative study. Am J Anat *171:* 149–161.

Palmiter, R.D., R.L. Brinster, R.E. Hammer, M.E. Trumbauer, M.G. Rosenfeld, N.C. Burnberg, S. Evans (1982) Dramatic growth of mice that develop from eggs micro-injected with metallothionein-GH fusion genes. Nature *300:* 611.

Pursel, V.G., R.E. Hammer, D.J. Bolt, R.D. Palmiter, R.L. Brinster (1990) Integration, expression and germ-line transmission of growth-related genes in pigs. J Reprod Fertil *41* (suppl): 77–87.

Slabaugh, M.B., M.E. Lieberman, J.J. Rutledge, J. Gorski (1981) Growth hormone and prolactin synthesis in normal and homozygous Snell and Ames dwarf mice. Endocrinology *109:* 1040–1046.

Takeuchi, T. (1956) Histochemical demonstration of phosphorylase. J Histochem Cytochem *4:* 84.

[*From the Proceedings of the Anatomical Society of Great Britain and Ireland*, 18–20 *July* 1984. J. Anat., (1984), **139**, p. 726]

27. Muscle cellularity and its relationship with birth weight and growth. By S. E. HANDEL and N. C. STICKLAND. *Departments of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh and The Royal Veterinary College, London*

A study of muscle growth and development was undertaken on the semitendinosus muscle of 36 Large White pigs of various ages. Frozen transverse sections were obtained from the muscle for histological and histochemical examination. At each selected age two littermates, categorised as the largest or smallest (less than 950 g) at birth, were used.

Low birth weight was associated with a reduced muscle fibre number which remained fixed during postnatal growth. The mechanism of this reduction has been elucidated prenatally (Wigmore & Stickland, *J. Anat.*, **137**, 1984) and this hypothesis has now been confirmed postnatally on a larger number of animals in this present study. The results verify that a reduced secondary-to-primary muscle fibre ratio in the pig of low birth weight, of approximately 11·0 % ($P < 0{\cdot}01$), is mainly responsible for the lower fibre number. These ratios were, on average, 25·5 and 22·7 in the large and small littermates respectively. Primary fibre number, which had a mean value of 17 844, was not significantly affected except in a subset of extreme cases. A severely low relative birth weight, when the 'small' pig was less than 50 % of the 'large' in weight at birth and was more than 2·5 S.D. from the mean litterweight, constitutes the exception. In this situation primary fibre number was up to 55 % less than in the largest littermate. The consequential growth potential of these animals was inevitably seriously limited.

The implications of these results on the distribution of myofibre types of m. semitendinosus and general body growth of large and small birth weight littermates are discussed.

Work supported by an AFRC grant.

XII International Anatomical Congress

A.271

THE ULTRASTRUCTURAL DEVELOPMENT OF TWO MYOFIBRE TYPES.

S. E. Handel and N. C. Stickland.

Department of Anatomy, The Royal Veterinary College.

London NW1 OTU, England.

Ultrastructural (electron microscopical) investigations are an ideal component of a comprehensive growth study, complimenting and augmenting light microscopical observations. Moreover, quantitative studies provide an invaluable reference in investigations of muscle pathology, aiding the early identification of myopathies, and enabling the evaluation of the rate and extent of their manifestation in the affected tissue. Despite this there is an apparent lack in the literature of comparative studies of the development of different myofibre types, from birth to maturity, which quantify the changes in different subcellular organelles.

An ultrastructural investigation into the postnatal development of oxidative (slow-twitch, oxidative metabolism) and non-oxidative (fast-twitch, glycolytic metabolism) fibres from the deep and superficial portions of m. semitendinosus, respectively, was therefore performed on 32 purebred Large White pigs from 11 litters. This study was undertaken to evaluate (by stereological analysis) changes in the mitochondrial, lipid droplet and myofibrillar percentage volumes of these two myofibre types between birth and 84 days of age, and to quantify differences between largest (mean birthweight of 1559g), smallest (mean birthweight of 1147g), and runt (mean birthweight of 758g) littermates. The subcellular organelles chosen were selected in order to ascertain, as well as the general morphological changes occurring in myofibres after birth, whether variations in birthweight between littermates resulted in any permanent difference in the myofibrillar protein content (a measure of muscle strength) or capacity for oxidative metabolism (as the mitochondrial and lipid droplet values would reveal) of their respective muscles.

The oxidative and non-oxidative fibres possessed, as would be expected, different compliments of mitochondria and lipid droplets; the high content of these organelles within the oxidative fibres prior to weaning was an apparent reflection of the high-fat diet. The oxidative and non-oxidative fibres, as well as presenting different myofibrillar compliments showed differential rates of myofibrillar accumulation. The relatively larger postnatal change in the percentage volume of myofibrils of the non-oxidative fibres, as opposed to the small change within the oxidative fibres, presented a cytological basis by which to explain the lack of sparing of the non-oxidative fibres, with respect to the oxidative fibres , to the effects of growth retardation. The ultrastructural composition of myofibres was not significantly different between littermates of disparate birthweight except when, as in two extreme cases, birthweight was severely reduced. In these instances the myofibrillar percentage volume of the non-oxidative fibres was greatly affected, once again illustrating the transient nature of myofibrillar protein content of the non-oxidative fibres of the young animal.

XII International Anatomical Congress

A.678

MUSCLE CELLULARITY IN LARGE AND SMALL BREEDS OF PIGS

N.C. Stickland and S.E. Handel

Department of Anatomy, The Royal Veterinary College

London NW1 0TU, England

During prenatal muscle development there is an initial formation of large primary myofibres. A larger population of smaller secondary myofibres then form around the primaries. It has been shown in many muscles that the primary myofibres take on slow-contracting characteristics and that the secondary myofibres take on fast-contracting characteristics (although some secondaries become slow during late prenatal and postnatal growth). These "metabolic bundles", derived from one primary myofibre and its surrounding secondaries, can be readily identified in postnatal pig muscle. Furthermore it has been shown that pigs which develop at disadvantaged sites in the uterus and which develop fewer muscle fibres in their muscles do so because fewer secondary myofibres form around each primary; the number of primary myofibres is not affected (1).

This present study was carried out in order to investigate whether genetically small animals develop fewer muscle fibres in their muscles by the same mechanism as in nutritionally small animals. The semitendinosus muscle was removed from eight commercial Large White pigs and from eight Miniature pigs. Complete frozen transverse sections were obtained for each muscle in its mid-belly region and stained for myosin adenosine triphosphatase activity; this enabled the identification of "metabolic bundles".

It was found that the Large White pigs had 173% more muscle fibres in their muscles than the Miniature pigs. The most important factor in bringing about this large fibre number difference was shown to be the number of primary myofibres contained in the muscles of the two breeds. Primary myofibre number was found to be about four times more important than secondary to primary myofibre ratios in determining muscle cell number in these genetically different sized animals.

1. Wigmore, P.M.C. & Stickland, N.C. (1983). Muscle development in large and small pig fetuses. J. Anat., 137, 235-245.

J. Anat. 152, 229

14. Skeletal muscle characteristics of Snell dwarf mice. By N. C. STICKLAND, PATRICIA E. MESCALL and A. R. CROOK. *Department of Anatomy, The Royal Veterinary College, London*

The Snell dwarf mouse (dw) is associated with impaired growth of the anterior pituitary resulting in reduced production of growth hormone and thyroid-stimulating hormone. It is known that administration of growth hormone can improve muscle growth in normal animals but changes in muscle due to genetically altered hormone levels have not been fully characterised.

Six Snell dwarf mice and six phenotypically normal littermates were used in this study and killed at 33 days of age. M. biceps brachii (a predominantly fast muscle) and m. soleus (a mixed fibre type muscle) were removed from each animal and complete frozen transverse sections obtained for all muscles. Serial sections were stained for various enzyme activities in order to identify muscle fibre types.

Analysis of the muscle sections revealed no difference in total muscle fibre number between the two groups of mice but there was a marked difference (particularly in m. soleus) in mean cross sectional area of the muscle fibres. The percentage of slow, oxidative fibres was approximately 32% in m. soleus for both groups of mice. However, there was a marked difference in the percentage of this fibre type in m. biceps brachii, being about 0·5% in the normal mice and 6·5% in the dwarf mice. Density of nuclei was approximately doubled in the muscle of dwarf mice although total nuclei numbers were half those found in normal mice. M. soleus exhibited greater differences in these parameters than m. biceps brachii.

The results presented here suggest that dwarfism affects only the postnatal growth of muscle with the slower muscle seemingly more affected than the fast muscle; there appears to be no lasting prenatal effect on muscle as far as the development of muscle fibre numbers is concerned.

The animals for this investigation were kindly provided by Dr A. T. Holder of the Institute of Child Health, London.

J. Anat. 167, 264

D. 9. The determinants of inter- and intra-litter variation in myofibre number in the pig. By C. M. DWYER and N. C. STICKLAND. *Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London.*

In the pig fetus muscle fibre hyperplasia occurs by the formation of primary and secondary fibres. The number of primary fibres which form is believed to be genetically determined, whereas the number of secondary fibres which form on each primary is more susceptible to environmental influences. The pig is a multiparous species and exhibits wide variation in size within and between litters. This study investigates the influence of genetic and environmental factors on this variation at the level of muscle cellularity both within and between litters.

Five Large White sows were mated and fed normally throughout pregnancy at levels currently used in pig production. Piglets were killed at 5 weeks and fresh, frozen sections of m. semitendinosus were taken and prepared for myosin ATPase histochemistry. The total number of muscle fibres, total number of primary fibres and the secondary-to-primary fibre number ratio were determined for each litter member ($n = 48$).

The results clearly showed the wide variation in myofibre number both between litters and between members of the same litter. Analysis of variance demonstrated that there was a significant variation in primary fibre number ($P < 0{\cdot}005$) between litters and this accounted for inter-litter variations in myofibre number since there was no difference in secondary-to-primary ratio between litters. Within individual litters, however, both the number of primary fibres and the secondary-to-primary ratio contributed to the variation in total fibre number. The more important factor determining intra-litter variation tended to be secondary-to-primary ratio in litters containing a large number of animals, or when the litter extremes were compared. In small litters or average litter members both factors seemed to make an important contribution.

These results suggest that the primary fibre number is less susceptible to environmental influences and therefore is a good indicator of the animal's genotype. The secondary-to-primary ratio, however, seems to be much more labile and is affected by environmental and nutritional factors.

J. Anat. 170, 234-235

19. The effects of genetic sex and androgens on skeletal muscle tissue. By N. C. STICKLAND and P. J. O'SHAUGHNESSY. *Department of Veterinary Basic Sciences, The Royal Veterinary College, London*

Previous studies have shown that certain muscles may exhibit different muscle fibre type proportions between the sexes. Experiments on the effects of either male castration or injection of androgens into female animals have led investigators to conclude that these differences are due to levels of androgens. However these conclusions are based on invasive techniques which may involve secondary factors, and results have usually only been apparent on a few highly 'androgen-sensitive' muscles. The sex-reversed (*Sxr*) mouse is genetically (X/X) female but phenotypically male due to the presence of the testis determining gene (*Tdy*). Serum testosterone in the *Sxr* male is in the low normal range and this animal provides the opportunity to examine whether factors other than androgens affect sexual dimorphism in muscle.

The biceps brachii (predominantly a fast muscle) and soleus (a slow muscle), neither of known high androgen-sensitivity, were removed immediately post mortem from ten normal (X/Y) males, ten normal (X/X) females, and ten (X/X) *Sxr* males. The muscles were sectioned and serial sections reacted for myosin adenosine triphosphatase, glycogen phosphorylase and succinic dehydrogenase activities. These tests enabled the fibre type proportions of each muscle to be assessed as well as the mean size of each fibre type.

In m. soleus the proportion of slow fibres in the *Sxr* male was similar to that in the normal male, being less than that in the normal female. Furthermore the mean sizes of each fibre type in the *Sxr* male were similar to the normal male, the fast fibres being larger and the slow fibres being smaller than the normal female. In m. biceps brachii only normal females contained a few slow fibres. However, in contrast to m. soleus, the fast fibres (of which there were two types in the biceps) were larger in the normal male than in both the *Sxr* male and normal female.

In conclusion, it would seem from this study that both muscles were sensitive to androgens with respect to fibre type differentiation but that one muscle (m. soleus) was more sensitive to androgens as far as muscle fibre growth was concerned. An alternative explanation is that genetic sex may be more important than androgen levels in determining muscle fibre growth in some muscles.

J. Anat. 173, 243-244

D.10. Preparation techniques for automatic counting of skeletal muscle fibres. By N. C. STICKLAND, CATHERINE M. SUTTON and A. R. CROOK. *Department of Veterinary Basic Sciences, the Royal Veterinary College, London*

Manual counting of skeletal muscle fibres in complete muscles is clearly a laborious technique. There are now several image-analysis systems available which may help with this task. However, for accurate counting the skeletal muscle fibres must be completely separated from their neighbours by a sufficiently different grey level. If the fibres are not separate the system will have difficulty in recognising them as separate objects.

Various techniques were attempted, on both frozen and wax-processed transverse sections, for visually separating muscle fibres. Techniques assessed included a reticulin stain, Masson's trichrome technique (which stains collagen and muscle fibres different colours), Sirius Red (stains collagen and basement membrane), and detection of fibronectin (present around all muscle fibres) using an antibody with peroxidase labelling. The best technique was found to be the staining of reticulin (with no counterstain) using a silver impregnation technique (based on Gomori, *Am. J. Path.* **13**, 1937) on frozen sections. The fresh, frozen sections were first fixed in buffered formal saline for 10 minutes, then washed in water before the reticulin technique was applied.

Automatic fibre counts were performed using the Seescan Image Analysis System (Cambridge, U.K.) on the reticulin-stained muscle sections. With no image editing there was a mean underestimate of 4·3 % when compared with manual counting. However, a great advantage of the reticulin stain is that fibre outlines are very obvious (more so than in any routinely stained

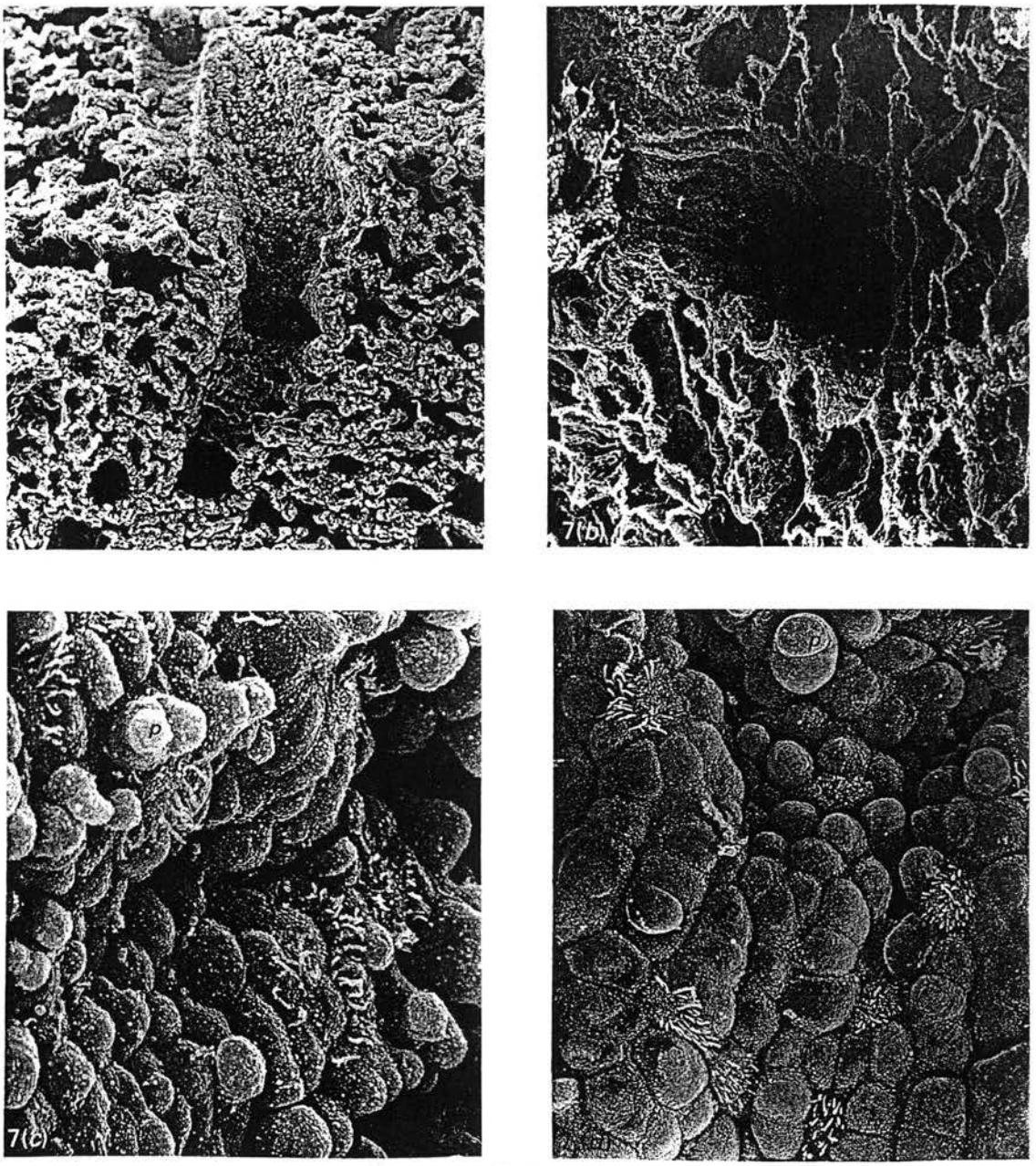

Fig. 7.

sections). Any errors of the system can therefore be readily detected and quickly corrected (adding only seconds to each sample time) to provide 100 % accuracy.

Anatomia Histologia Embryologia 20: 278

STICKLAND, N. C., A. R. CROOK, and C. M. SUTTON (London, U. K.): **Divergent Specialisations of Skeletal Muscle Fibres in Cats and Dogs.**

M. semitendinosus was removed immediately post mortem from a range of cats and dogs, both wild and domestic. The muscles were rapidly frozen and transverse sections taken at the mid-belly level. Histochemical tests were performed on these sections; tests included myosin ATPase activity, succinic dehydrogenase and glycogen phosphorylase activities. Using these tests muscle fibres could be classified as slow oxidative (SO), fast oxidative glycolytic (FOG) or fast glycolytic (FG). The proportions and cross-sectional areas of each fibre type were estimated for each animal.

The results showed that wild cats have a relatively greater slow muscle fibre content in their muscles than either domestic cats, wild dogs or domestic dogs. This result may be associated with the greater body weight of the wild cats used in this investigation. A more important result is that all muscle fibres in wild and domestic dogs exhibited oxidative metabolism. In contrast, the great majority of fast muscle fibres in wild and domestic cats exhibited non-oxidative, glycolytic metabolism. This result can be related to the different locomotory behaviour of dogs and cats.

J. Anat. 176, 267

D.28. Localisation of IGF-1 and IGF-2 in a range of vertebrate skeletal muscle tissue. By S. C. F. RAWLINSON, M. L. USHER*, C. M. DWYER and N. C. STICKLAND. *Department of Veterinary Basic Sciences, The Royal Veterinary College, London, and * Department of Physiology, University College London*

Antibodies to Insulin-like Growth Factors (IGF) 1 and 2 were used in an immunocytochemical approach to the localisation of IGF-1 and IGF-2 in sections of vertebrate skeletal muscle tissue. The aim of the investigation was to develop the technique and to test its applicability to various vertebrate muscles as a prerequisite to further investigations.

The antibodies were polyclonal, raised in rabbit (for IGF-1) or rat (for IGF-2) against human IGFs. Muscles used were m. tibialis anterior from chick embryos (18 days *in ovo*), m. lateralis from juvenile carp, and m. biceps brachii and m. soleus from guinea-pigs, both fetal (50 days' gestation) and postnatal (six weeks). The muscles were removed immediately post mortem, chilled by precipitate immersion in Arcton (ICI, Ltd) cooled in liquid nitrogen, and sectioned transversely in a cryostat. 10 μm-thick sections were air dried, and fixed in 3·7% formaldehyde in phosphate-buffered saline (PBS) at pH 7·4 for one hour, followed by washing ($\times 3$) in PBS. The sections were background blocked in 5% heat inactivated goat serum for one hour. Primary antibody was then applied for one hour before washing ($\times 3$) in PBS. This was followed by secondary antibody application (either FITC or TRITC, Sigma) for 40 minutes. Sections were finally washed in PBS ($\times 3$) and mounted in PBS:glycerol (1:9) including 5% propylgallate. Sections were viewed under UV illumination.

The localisation of IGF-1 appeared to be similar for all muscle samples with a homogeneous distribution within all muscle fibres but with evidence of some concentration within the periphery of muscle fibres in the fetal guinea-pig. The pattern of IGF-2 distribution was not as clearly defined although, in the chick and fetal guinea-pig, there again appeared to be a concentration within the periphery of some muscle fibres as well as some inter-fibre localisation.

The IGF antibodies were kindly supplied by Dr D. J. Baylink (Veterans' Hospital, Loma Linda, California, USA).

J. Anat. 179, 217

18. Skeletal muscle in the genetically obese mouse (genotype, *ob/ob*). By N. C. STICKLAND, R. A. L. BATT, A. R. CROOK, and C. M. SUTTON. *Department of Veterinary Basic Sciences, The Royal Veterinary College, London*

The genetically obese mouse is up to three times heavier than its phenotypically normal (wild-type) littermate. Many features significantly affected in the obese mouse may be 'normalised' by a limited feeding regime designed to prevent the excessive weight gain. The present study comprised two experiments designed to test the hypothesis that there are differences in skeletal muscle characteristics between wild-type and obese mice which can be attributed to the enormous difference in body weight.

The first experiment involved three groups of mice: (*a*) wild type fed *ad lib.* (*b*) obese fed *ad lib.* and (*c*) obese limit-fed (for each group $n = 5–9$). Mice in the latter group were limit-fed such that by 1 year of age they were not significantly different in body weight from the wild types. All animals were killed by decapitation at approximately 1 year of age and 5 muscles were removed from each. All 5 muscles were significantly heavier in the wild-type animals; muscle weight in the 2 obese groups did not differ. Furthermore, there was a significant correlation between muscle weight and body weight within the wild-type group but not in either of the obese groups.

The second experiment was designed to investigate the histochemical characteristics of the muscles. In addition to the 3 groups of mice used in the first experiment, an obese group was reduced in weight dietarily. Mice (all female; $n = 6–10$) were killed at approximately 9 months of age and m. biceps brachii and m. soleus were removed and prepared for histochemistry. There was no marked difference in the total number of muscle fibres or in proportions of muscle fibre types between the groups apart from a reduction in the proportion of slow, oxidative fibres in the soleus muscle of the weight-reduced obese mice.

Arguably the reduced muscle weight in obese mice fed *ad lib.* could be attributed to their posture and reduced activity. However, the inability of muscles of limit-fed animals (which enabled increased activity) to increase in weight suggests that other factors are involved and that the effects on muscle may be a primary genetic effect rather than a secondary one.

POSTNATAL GROWTH IN THE PIG AND ITS RELATIONSHIP TO MUSCLE FIBRE NUMBER.

C. M. Dwyer, J. Fletcher *, & N. C. Stickland
Royal Veterinary College, University of London, Royal Coll. St., London NW1 0TU
*Unilever Research Colworth Laboratory, Sharnbrook, Beds.

Fast growing strains of many species, including the pig, are known to have more muscle fibres than slower growing strains (Ezekwe & Martin, 1975; Stickland & Handel, 1986). These observations have been extended by examining the growth of pigs within the same strain.

This project involved seven litters of Large White x Landrace pigs which were bred and reared under commercial conditions. Piglets were weighed at birth and at monthly intervals during growth. Pigs were killed at approximately 80 kg and the semitendinosus muscle was dissected out from the left side and weighed. A whole mid-belly slice was cut and prepared for histochemistry. Sections of 10 μm thickness were cut and stained to demonstrate myosin ATPase activity, using a modification of the method of Guth and Samaha (1970). An estimation of total fibre number was made for each animal (n = 66). In addition, estimations of primary fibre number and secondary-to-primary fibre number ratio (S : P ratio) were made for each muscle in order to assess the relative contributions of nutritional and genetic factors (Dwyer & Stickland, 1991).

Piglet growth could be divided into three main phases : from birth to 6 kg, from 6 kg to 25 kg, and from 25 kg until slaughter. The first two growth phases were significantly correlated with birth weight ($r = 0.3614$, $P < 0.05$ and $r = 0.5873$, $P < 0.001$, respectively) but were not correlated with total muscle fibre number. In the third growth phase, however, when the pigs were growing most rapidly, the average daily gain (ADG) in weight was significantly correlated with muscle fibre number ($r = 0.4149$, $P < 0.001$) and was not correlated with birth weight. Primary fibre number and S : P ratio were both significantly correlated with muscle fibre number ($r = 0.7642$ and $r = 0.4093$, $P < 0.001$, respectively), however only S : P ratio was correlated with ADG ($r = 0.4393$, $P < 0.001$). In addition the feed / gain ratio was calculated and found to have a negative correlation with muscle fibre number

during this period of growth ($r = -0.4571$, $P < 0.001$).

These results suggest that muscle fibre number is an important determinant of post natal growth, such that pig littermates with a high muscle fibre number tend to grow faster and more efficiently than littermates with a lower fibre number.

Dwyer & Stickland (1991) Anim. Prod. **52** : 527 - 533
Ezekwe & Martin (1975) Growth **39** : 95 - 106
Guth & Samaha (1970) Exp. Neurol. **28** : 365 - 367
Stickland & Handel (1986) J. Anat **147** : 181 - 189

3. Fish and avian muscle development, growth and adaptation

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Relationship Between Size of Muscle Fibres and Body Dimensions in a Number of Teleosts

In 1956, JOUBERT[1] reviewed some of the literature which dealt with the effect of species on muscle fibre size. He quoted BOWMAN[2], who in 1840, must have been one of the first to suggest some sort of genetic control over muscle fibre size. He stated that within each class of animals there is an extensive range of body size and probably also muscle fibre size. Since then, several studies have been made to investigate the effect of species on muscle fibre size within the mammalian class. WARRINGSHOLZ[3], and later JOUBERT[1], measured muscle fibre diameter in 4 domestic mammals, but they found no relationship with body size. However, GAUTHIER and PADYKULA[4], in a more recent extensive survey, did establish a direct relationship between fibre size in the diaphragm of 13 mammalian species and body size. GEORGE and NAIK[5], working on birds, also found that muscle fibre area increased with body weight. This last study seems to be the only one concerned with investigating the relationship between fibre size and body size outside the mammalian class. It was decided, therefore, to investigate this relationship (if any) in fish, which have so far not been studied and which also have an easily measured 'shape' in contrast to mammals.

Materials and methods. 17 different species of teleost (Table I, identified according to SMITH[6]) were caught over a 3-day period from rock pools found in the exposed coral reef off the Kenya coast (4°20′S). The fish were stunned by a blow on the head and then fixed in 5% formalin for 24 h with a small dorsal and ventral skin incision to aid penetration of fixative. After fixation, the length (l) of the fish was measured (from snout to base of tail) as well as its greatest height (h) and width (w) (using microcalipers). The fish were then decalcified, according to SCHAFFER[7], in order to make sectioning easier. The whole fish was sectioned transversely at a position about two-thirds of its length from the head end; 7 µm sections were cut on a Leitz rotary microtome and stained in Haematoxylin and Eosin.

By means of an eye-piece gracticule in a Leitz microscope, 100 muscle fibres were measured[8] for each fish. The approximate 'volume' (V) of each fish was estimated using the following equation:

$$V = \frac{1}{3} \cdot \pi \cdot \frac{h \cdot w \cdot l}{4}$$ (see above for explanation of symbols)

This equation is the volume of 2 elliptical cones (total length, l) with their bases end to end. This will give some indication, at least, of relative volumes of the fish used.

It was found that the lengths of the fish used were on average 29.9% (with a standard error of ± 4.2) of the maximum length they can attain (according to SMITH[6]). In order to standardize the results, it was decided to adjust all Volume and Fibre diameter measurements to their estimated values at 30% of the maximum length of the fish. These were very small adjustments, which could easily be carried out assuming that, for volume changes, length:height:width ratios are constant for each species. Muscle fibre diameter adjustments were based on the fact that changes in fibre diameters are related to changes in overall muscle girth[9].

Results. Table I lists all the fish used in this investigation together with the mean muscle fibre diameter (D), 'volume' (V) and length:maximum height ratio (l/h) for each fish. The parameters D and V shown in this table have both been 'adjusted' to 30% of the fish's maximum length. Table II shows the coefficients of correlation between various parameters. All coefficients in this table are based on the 'adjusted' values shown in Table I. In some correlations, fish number 13 was omitted as it appears to be very different in fibre size and volume from the other fish (see Figure). It should be noted that, using original values for Volume and Fibre diameter (with *no* adjustment to 30% of the fish's maximum length), correlation coefficients were significant for D against log(V) ($r = 0.68$, $p < 0.01$) and for D against l/h ($r = 0.57$, $p < 0.02$). Other correlations were of the same order as Table II but not significant. The Figure shows the variation of muscle fibre diameter with volume and length:height ratio of the fish.

Table I. List of fish used in investigation with mean muscle fibre diameter (D), estimated body volume (V) and length:height ratio (l/h)

No.	Name	D (µm)	V (c.c.)	l/h
1	*Canthigaster margaritatus*	44.3	2.94	2.38
2	*Scorpaena mossambica*	42.3	1.72	2.42
3	*Stethojulis strigiventer*	27.2	0.71	3.18
4	*Halichoeres dianthus*	16.0	0.38	2.96
5	*Halichoeres scapularis*	34.9	1.23	4.27
6	*Caracanthus zeylonicus*	48.0	2.41	2.77
7	*Abudefduf lacrymatus*	25.1	1.31	2.13
8	*Cheilodipterus quinquelineatus*	35.4	0.95	2.94
9	*Branchiostegus japonicus*	48.6	1.70	3.53
10	*Abudefduf sparoides*	51.5	1.31	1.85
11	*Apogon monochrous*	49.5	0.94	2.51
12	*Gobiodon citrinus*	15.1	0.04	4.07
13	*Siganus oramin*	88.7	20.18	2.26
14	*Apogonichthyoides uninotatus*	28.6	0.67	3.32
15	*Petroscirtes mitratus*	21.9	0.11	8.51
16	*Acropoma japonicum*	23.3	0.30	3.16
17	*Grammistes sexlineatus*	47.9	8.44	2.63

Table II. Coefficients of correlation (r) between various parameters[a]

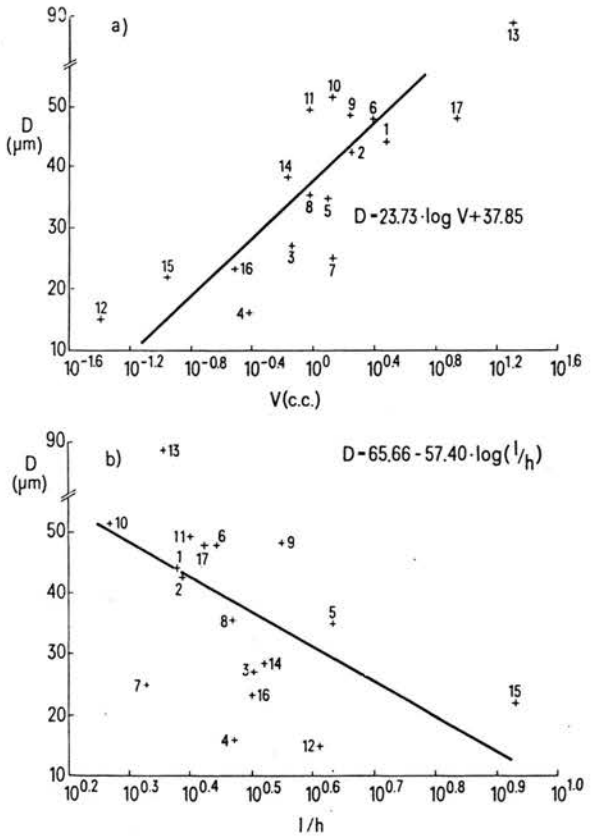
Parameters correlated	n	r	P
D v. V	17	0.814	< 0.001
D v. log (V)	17	0.844	< 0.001
D v. V (13 omitted)	16	0.536	< 0.05
D v. log (V) (13 omitted)	16	0.765	< 0.001
D v. l/h	17	−0.412	n.s.
D v. log (l/h)	17	−0.482	< 0.05
D v. l/h (13 omitted)	16	−0.432	n.s.
D v. log (l/h) (13 omitted)	16	−0.493	< 0.1

[a] D, muscle fibre diameter; V, volume of fish; l/h, length: height ratio.

Discussion. The results of this investigation appear to show that the diameter of muscle fibres in various species of fish is related in some way to the volume of the fish. Larger species of fish seem to have larger muscle fibres. The fact that the correlation coefficients are highest when log (Volume) is used, seems reasonable when it is realized that the volume parameter is a cubed dimension as compared to the diameter measurement. As already mentioned, this relationship between fibre size and body size has already been demonstrated in several species of birds by GEORGE and NAIK[5]. SMITH[10], working with chickens, also concluded that muscle size is related to body size.

In mammals, although this relationship has been demonstrated by GAUTHIER and PADYKULA[4] using 13 species, other workers[1,3] have not been able to show this, even though the later workers used smaller numbers of species. This discrepancy in the mammalian class may be due in part to the much greater range of body sizes and dimensions than that found in the bird or fish class. In fact, if certain groups of mammals are treated separately, a direct relationship between fibre size and body size is seen. JULIAN and CARDINET[11] found that large dogs had larger muscle fibres than small dogs, and LUFF and GOLDSPINK[12] found a similar stituation in mice.

The results of this investigation also suggest that there is an inverse relationship between muscle fibre size and length:height ratio (l/h). This relationship becomes statistically significant when log (l/h) is used, suggesting that the relationship is not linear.



Shows the relationship between mean muscle fibre diameter (D) and (a) Volume of fish (V), (b) length: height ratio (l/h). Both V and l/h axes are written on a log scale. The equations for the plotted lines of regression are shown. The numbers against each fish correspond to the fish numbers shown in Table I.

There is some evidence[13] that faster fish tend to have a higher length:height ratio. It would seem, therefore, that faster fish may have smaller fibre diameters. In mammals, GAUTHIER and PADYKULA[4] reasoned that fibre size is related to body size because body size is inversely related to metabolic activity[14]. Only if metabolic activity in fish is related to relative speed can it be said that the results presented here agree with those found in mammals.

In mammals, the decrease in fibre size with increasing metabolic activity is due to a higher proportion of red muscle fibres, as well as a decrease in size of the white muscle fibres[4]. In the fish studied here, the characteristic lateral line strip of red muscle fibres[15] appeared very small or non-existent, and, in any case, these fibres were not included in the measurements. However, in some of the 'faster' fish, a 'mosaic' arrangement of small and large fibres was seen in the bulk of muscle which might correspond to the mosiac arrangement of red and white muscle fibres seen in some fish[15]. A histochemical study would be required to investigate this idea.

Taken as a whole, the results pose difficult problems. If muscle fibre size is directly related to body size and inversely related to l/h then this suggests that smaller fish are more stream-lined with smaller muscle fibres. It is possible that the relationship between fibre size and l/h is a result of a relationship in the fish used here between body size and l/h. A wider range of teleosts should be examined, preferably in which body size and l/h are unrelated, in order to investigate these inter-relationships more fully[16].

Summary. In a survey of 17 species of teleosts, a direct relationship was found between the diameter of muscle fibres and estimated volume of the fish. The results also suggested an inverse relationship between muscle fibre diameter and 'streamlinedness' of the fish (as measured by length:height ratio).

N. C. STICKLAND

Department of Veterinary Anatomy and Histology, University of Nairobi, P.O. Box 30197, Nairobi (Kenya), 2 June 1975.

[1] D. M. JOUBERT, J. agric. Sci. *47*, 59 (1956).
[2] W. BOWMAN, Phil. Trans. *130*, 457 (1840).
[3] C. W. WARRINGSHOLZ, Arch. wiss. prakt. Tierheilk. *29*, 5 (1903).
[4] G. F. GAUTHIER and H. A. PADYKULA, J. Cell Biol. *28*, 333 (1966).
[5] J. C. GEORGE and R. M. NAIK, J. Anim. Morph. Physiol. *6*, 90 (1959).
[6] J. L. B. SMITH, *The Sea Fishes of Southern Africa*, 5th edn. (Central News Agency, South Africa 1965).
[7] J. SCHAFFER, Z. wiss. Mikrosk. *19*, 308 and 441 (1902).
[8] N. C. STICKLAND, Ph. D. Thesis, University of Hull (1973).
[9] N. C. STICKLAND and G. GOLDSPINK, Anim. Prod. *16*, 135 (1973).
[10] J. H. SMITH, Poultry Sci. *42*, 283 (1963).
[11] L. M. JULIAN and G. H. CARDINET, Anat. Rec. *139*, 243 (1961).
[12] A. R. LUFF and G. GOLDSPINK, Life Sci. *6*, 1821 (1967).
[13] P. GREENWAY, Experientia *21*, 489 (1965).
[14] F. G. BENEDICT, *Vital Energetics. A Study in Comparative Basal Metabolism* (Carnegie Inst. Washington Publ. 1938), No. 503.
[15] R. BODDEKE, E. J. SLIJPER and A. VAN DER STELT, Proc. K. ned. Akad. Wet., Series C. *62*, 589 (1959).
[16] Thanks are due to Mr. THEO D'SOUZA for help in identifying the fish used in this investigation.

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Succinic Dehydrogenase Distribution in the Pectoralis Muscle of Several East African Birds

N. C. Stickland

Department of Veterinary Anatomy and Histology, University of Nairobi, Kenya
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Abstract

The distribution of succinic dehydrogenase activity was investigated in the pectoralis muscle of thirteen East African birds, representing five Orders. It was found that the pectoralis muscle of the most primitive birds studied (Galliformes) contained all "white" muscle fibres whereas the more advanced birds (Passeriformes) had all "red" muscle fibres. Intermediate Orders had mostly a mixture of red and white muscle fibres. There also appeared to be a direct relationship between body size and average muscle fibre size. However, it was concluded that the most important factor in relation to the muscle structure is the bird's mode of flight. The relationship with the degree of evolution and body size only held true in so far as the birds which had developed the facility for sustained flight, by increasing their red muscle fibre content, were also smaller in size and constituted the more "evolved" Orders of birds. In support of this it was noted that migratory birds (i.e. engaging in sustained flight) from more primitive Orders also had a high red muscle fibre content in their pectoralis muscles.

Introduction

Based upon a classification of muscle fibres into red, white or intermediate nearly all combinations of muscle fibre types have been found in the avian pectoralis by George and his associates (e.g. Chandra-Bose and George 1965, George and Talesara 1962). The results of this work suggest that there is an evolutionary trend in the avian pectoralis towards the all red muscle fibre type (George and Berger 1966). However, apart from this work on Indian birds and a little work on North American birds by Salt (1963), no such work on the characterisation of muscle fibre types in the avian pectoralis has been carried out on birds from any other country. It was therefore felt that, owing to the large range of birds available in East Africa, such a study in this country might help to further the understanding of the cellular organisation of the avian pectoralis and perhaps lend support to the theory mentioned above.

Materials and Methods

Several species of East African birds (listed in Table 1), which had been caught in nets, were killed by dislocation of the neck and small pieces of pectoralis muscle were immediately removed near the anterior border of the sternum. The muscles were placed on a Leitz freezing microtome stage (knife and stage set at —20°C) with rapid freezing aided by spraying the muscle with liquid Arcton 12 (ICI Limited, England). 15 μm transverse sections were cut and then treated to show the distribution of succinic dehydrogenase activity by the method of Nachlas et al. (1957). Sections were also stained for fat using Sudan IV.

By means of a Leitz projection microscope the number of muscle fibres (red, white and total) per mm^2 was counted, using the sections stained for succinic dehydrogenase activity. Using the same projected image the areas of the cross sections occupied by red and white muscle fibres were outlined on white paper and the area ratios estimated by paper weight ratios. The average size of the muscle fibres was estimated by measuring the diameters of one hundred fibres of each type, using a calibrated eye-piece graticule.

It should be noted here that a "red" muscle fibre in this present investigation was one high in succinic dehydrogenase activity and a "white" muscle fibre was one with low activity. All sections underwent identical treatment with equal incubation times so that different birds could be compared.

Table 1. *Muscle fibre characteristics in the pectoralis muscle of birds used in this investigation*

	Length (in.)	Muscle fibres				
		No./mm.²	No. Red : White (%)	Area Red : White (%)	Size (μm) Red	Size (μm) White
Galliformes						
Numida mitrata (Helmeted Guinea-fowl)	20—22	210	0 : 100	0 : 100	—	70.4
Pternistis leucoscepus (Yellow-necked Spurfowl)	13—14	318	0 : 100	0 : 100	—	54.2
Francolinus afer (Grey-wing Francolin)	11	298	0 : 100	0 : 100	—	58.5
Gruiformes						
Eupodotis senegalensis (White-bellied Bustard)	24	926	90.4 : 9.6	81.7 : 18.3	27.0	52.3
Charadriiformes						
Calidris minuta (Little Stint)	5—5 ½	1960	100 : 0	100 : 0	26.5	—
Pterocles exustus (Chestnut-bellied Sandgrouse)	12	843	80.7 : 19.3	63.0 : 37.0	25.4	51.9
Columbiformes						
Streptopelia senegalensis (Laughing Dove)	9 ½	634	90.4 : 9.6	80.6 : 19.4	38.8	59.5
Streptopelia capicola (Ring-necked Dove)	10	861	86.8 : 13.2	67.2 : 32.8	24.5	57.0
Columba livia (Blue Rock Pigeon)*		885	91.0 : 9.0	73.5 : 26.5	31.4	50.1
Passeriformes						
Ploceus cucullatus (Black-headed Weaver)	7	1084	100 : 0	100 : 0	31.4	—
Coliuspasser ardens (Red-collared Widow-bird)	5	1023	100 : 0	100 : 0	31.9	—
Quelea quelea (Red-billed Quelea)	5	1144	100 : 0	100 : 0	31.3	—
Estrilda astrild (Waxbill)	4 ½	1040	100 : 0	100 : 0	27.7	—

* Not strictly an East African bird, but included for comparisons with work of other researchers.

Results

Table 1 lists all the birds used in this investigation and shows all the measurements made on the muscle sections. The birds were identified and named according to Williams (1963). The length shown for each bird is the average length from tip of bill to tip of longest tail feather. This information is also taken from Williams (1963) and is included to give some indication of the size of the bird. The birds are listed in their Orders from the most primitive (Galliformes) to the most advanced (Passeriformes).

The results in Table 1 were obtained from sections reacted for succinic dehydrogenase activity. The sections stained for fat, however, showed a similar picture with the "red" fibres (high succinic dehydrogenase activity) being high in fat content and the "white" fibres being low, although the distinction was often not so clear in the fat-stained sections.

The results in Table 1 show that the pectoralis muscles of the Galliformes contain all large "white" fibres whereas the Passeriformes have an all "red" fibre pectoralis. The intermediate Orders appear to have a mixture of both red and white muscle fibres.

Discussion

It can be seen from Table 1 that the large Galliformes have a pectoralis muscle characterised by a population of large muscle fibres whereas the smaller Passeriformes are characterised by narrow muscle fibres. The intermediate-sized birds have a mixture of large and small fibres so that the average muscle fibre size is also intermediate. It should be pointed out that the Little Stint, a small bird, although in one of the intermediate Orders, has only narrow muscle fibres the same as the other small birds. The largest bird used in this investigation is the Helmeted Guinea-fowl which has by far the largest muscle fibres. The White-bellied Bustard, although longer than the Helmeted Guinea-fowl by virtue of its relatively long neck, is by no means heavier which helps to explain its smaller muscle fibre size. Further possible reasons for this are, however, discussed below.

This relationship between muscle fibre size and body size which this present investigation appears to demonstrate has also been shown by Gauthier and Padykula (1966) in the mammalian diaphragm, in so far as body size was inversely related to metabolic rate and breathing frequency, and by Stickland (1975) in fish. This relationship was first suggested in birds by George and Naik (1959) so that this present investigation appears to support this theory.

When considering the muscle fibre types in this investigation it would seem that the results support the theory (George and Jyoti 1955, George and Berger 1966) that there is an evolutionary trend from the broad "white" muscle fibre to the narrow "red" muscle fibre type. This trend is shown in the five Avian Orders represented here from the primitive Galliformes to the advanced Passeriformes. The Little Stint is perhaps an exception here in that its pectoralis muscle contains all "red" muscle fibres although it is placed in a fairly primitive Order. The over-riding factor here is that this bird is a palaearctic migrant (none of the other birds in this investigation are migrants) and so would need a high content of red muscle fibres for long-distance flying. The White-bellied Bustard might be considered as having a large proportion of red muscle fibres for a bird in such a primitive Order. This may possibly be explained by the fact that other European Bustards are in fact migratory and as all Bustards would have followed the same evolutionary path the local Bustard would also have the same pectoralis muscle fibre characteristics. It should be mentioned here that the classification of muscle fibres in this investigation into red and white has been based on mitochondrial succinic dehydrogenase activity. Classification of muscle fibres is strictly more complicated than this, as put forward by Davies and Gunn (1972) amongst others. It is indeed conceivable that the white muscle fibres of the Galliformes used here could be Intermediate muscle fibres although they did appear to have the same level of activity as the white muscle fibres in the other Orders.

From the foregoing discussion it can be concluded that, although muscle fibre size seems to be related to body size and muscle fibre type proportions seem to be related to the level of evolution, the important factor is the development of the bird's mode of flight. Hence, the relationships of muscle fibres with body size and evolution, only hold true by virtue of the fact that birds in the more evolved Orders have generally become smaller in size and have developed a more advanced mode of flight, being capable of sustained flight, whereas the more primitive birds with 'whiter' muscle fibres can only fly short distances. It is worth pointing out

here that the inconsistencies of one or two of the so-called 'primitive' birds having a high content of red muscle fibres may be due, in part, to a present inadequacy in the classification of some birds.

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References

Chandra-Bose, D. A., George, J. C. 1965. Studies on the structure and physiology of the flight muscles of birds. 13. Characterization of the avian pectoralis. *Pavo 3:* 14—22.

Davies, A. S., Gunn, H. M. 1972. Histochemical fibre types in the mammalian diaphragm. *J. Anat. 112:* 41—60.

Gauthier, G. F., Padykula, H. A. 1966. Cytological studies of fibre types in skeletal muscle. A comparative study of the mammalian diaphragm. *J. Cell Biol. 28:* 333—354.

George, J. C., Berger, A. J. 1966. *Avian Myology.* Academic Press, New York, London. 500 pp.

— *Jyoti, D.* 1955. Histological features of the breast and leg muscles of bird and bat and their physiological and evolutionary significance. *J. Animal Morphol. Physiol. 2:* 31—36.

— *Naik, R. M.* 1959. Studies on the structure and physiology of the flight muscles of birds. 6. Variation in the diameter of the fibres of the pectoralis major and its relation to the muscle size and mode of flight. *J. Animal Morphol. Physiol. 6:* 90—94.

— *Talesara, C. L.* 1962. Histochemical demonstration of certain oxidizing enzymes in the pectoralis major muscle of the Rosy Pastor (*Pastor roseus*), Goose (*Anser albifrons*) and Fowl (*Gallus domesticus*). *J. Animal Morphol. Physiol. 9:* 59—62.

Nachlas, M. M., Tsou, K., de Souza, E., Cheng, C., Seligman, A. M. 1957. Cytochemical demonstration of succinic dehydrogenase by the use of new p-nitro-phenyl substituted ditetrazole. *J. Histochem. Cytochem. 5:* 420—436.

Salt, W. R. 1963. The composition of the pectoralis muscles of some passerine birds. *Can. J. Zool. 41:* 1185—1190.

Stickland, N. C. 1976. Relationship between size of muscle fibres and body dimensions in a number of teleosts. *Experentia 31.* 1279—1281.

Williams, J. G. 1963. *A Field Guide to the Birds of East and Central Africa.* Collins, London. 288 pp.

Dr N. C. Stickland
Department of Anatomy
Royal (Dick) School of Veterinary Studies
Edinburgh EH9 1QH
Scotland, U.K.

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Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*)

N. C. STICKLAND

Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH

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INTRODUCTION

It is now generally accepted that, in mammals, muscle hypertrophy during normal postnatal growth is due almost exclusively to muscle fibre hypertrophy, with very little contribution from fibre hyperplasia (Rowe & Goldspink, 1969) except, perhaps, in the very early postnatal period in some animals (Rayne & Crawford, 1975).

The far fewer studies that have been carried out on muscle growth in fish suggest, however, that hyperplasia may be an important factor in muscle hypertrophy during postlarval growth (Greer-Walker, 1970; Weatherley, Gill & Rogers, 1979) although not all studies are in agreement with this (Kryvi & Eide, 1977). Some of the discrepancies may be due to the fact that the fish are not usually studied throughout their whole period of growth, and it is quite likely that the relative contributions of fibre hypertrophy and hyperplasia to muscle growth change during the lifespan of the fish, as is the case in prenatal muscle development in mammals (Stickland, 1981).

As in most teleost fish the lateralis muscle of the rainbow trout can be divided into a superficial layer, near the lateral line, of red fibres and, beneath this, a far larger mass of white fibres. Within the large mass of white muscle there are small diameter fibres which give the muscle a mosaic appearance (Boddeke, Slijper & Van der Stelt, 1959), but these small fibres were shown by Johnston, Ward & Goldspink (1975) to have the same histochemical properties as the large white fibres. Weatherley, Gill & Rogers (1980) suggest that these small fibres are a stage in the development of the larger white fibres. The lateralis muscle of the rainbow trout does therefore provide a means of studying the growth of red and white muscle fibres separately.

The present investigation was carried out in order to estimate the contribution of fibre hyperplasia and hypertrophy to the growth of red and white muscle in the rainbow trout during the whole period of postlarval growth.

MATERIALS AND METHODS

Seventeen rainbow trout (*Salmo gairdneri*) ranging in length from 2·2 cm (immediately postlarval) to 71 cm (approximately the normal maximum size of rainbow trout) were obtained from Hopewood Trout Farm, Peebles. Immediately after killing the fish by a sharp blow to the head, a complete transverse slice of lateralis muscle was removed from one side of the fish at a level two thirds of the length from the head end. This slice was frozen in dichlorodifluoromethane (Arcton 12, I.C.I.), which was cooled to its melting point (−158 °C) with liquid nitrogen. The slices were then cut at −20 °C, using a cryostat, in order to obtain 20 μm transverse

sections of the complete lateralis muscle (red and white components) from one side. The sections were stained with haematoxylin and eosin. Low power photomicrographs of whole sections were made, as well as high power photomicrographs of red muscle and three regions of white muscle dorsal to the lateral line, namely, superficial, middle and deep.

A Videoplan image analyser (Reichert-Jung) was used to estimate the cross sectional area of the red and white muscle portions in the complete sections. The analyser was also used to measure 100 fibres from each of the high power photomicrographs of red muscle and superficial, middle and deep white muscle. The measurements obtained were fibre cross sectional areas (fibre areas) and diameters of circles with equivalent areas (fibre diameters). For the superficial white muscle measurements, the two or three most superficial layers of fibres were not included in the measurements because these are known to have slightly different properties and to be generally smaller than the remainder of the white muscle, being known as pink fibres (Johnston *et al.* 1975).

The fibres in the high power photomicrographs were counted so that estimates of the number of fibres per unit area were obtained for both red and white muscle. These estimates, together with the red and white muscle cross sectional area measurements, were used to calculate the total number of fibres in the complete sections. Enough photomicrographs were used to ensure that approximately 5 % of the total number of fibres were counted.

The effects of growth on fibre size (both cross sectional area and diameter) and fibre number were examined by plotting these parameters against fish length. Fibre diameter measurements were used so that a comparison could be made with the results of several other workers who have used this parameter. Relationships were defined by regression equations, using simple transformations (e.g. log, power, exponential) as necessary to find the best-fit regression line.

RESULTS

The relationships between the cross sectional areas of red and white muscle from one side of the fish and fish length are shown in Figure 1. The regression coefficients of the two power curves (Table 1) are not significantly different, which indicates that red and white muscle increase in cross sectional area at the same rate throughout growth (as measured by fish length increase). The ratio of cross sectional area of white muscle to that of red muscle remained at about 25 : 1 throughout growth.

The increase in the total number of red muscle fibres and white muscle fibres (from one side of the fish) with increasing fish length is shown in Figure 2. Table 1 shows that for the white muscle the best fit curve is a polynomial, indicating (Fig. 2) that fibre number increases rapidly at first and then slowly, reaching a plateau, at about 65 cm, of 150000 muscle fibres. The relationship between red fibre number and length is much more variable and none of the regressions attempted (logarithmic, power, exponential, various degree polynomials) was significantly better than the linear regression (Table 1) shown. Owing to the large amount of variability, however, (indicated by the high S_b value) it is not possible to state whether the red fibre number rate of increase changes during growth, although there is clearly an overall increase.

The increases in mean fibre cross sectional area for red and white muscle with length (Fig. 3) are exponential in form (Table 1). This means that for every 20 cm

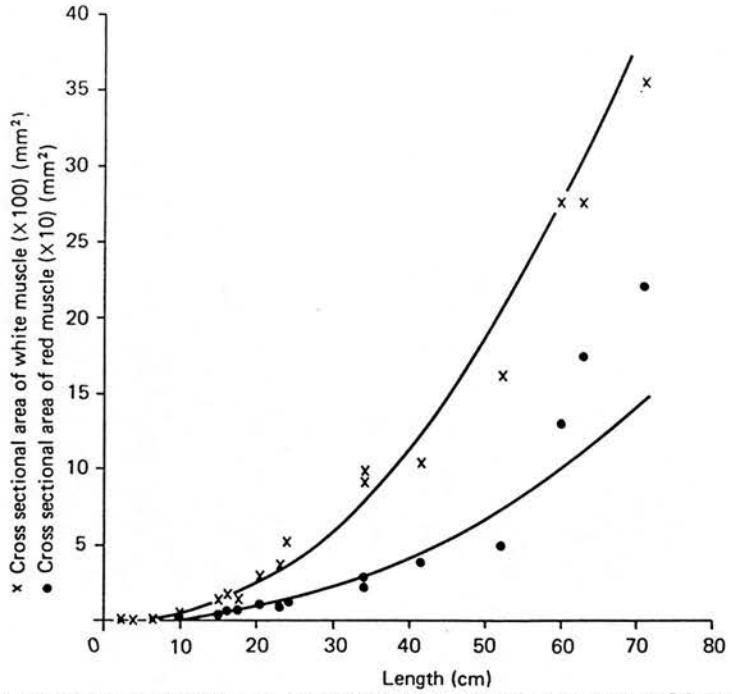

Fig. 1. Total cross sectional area of white muscle and red muscle versus fish length.

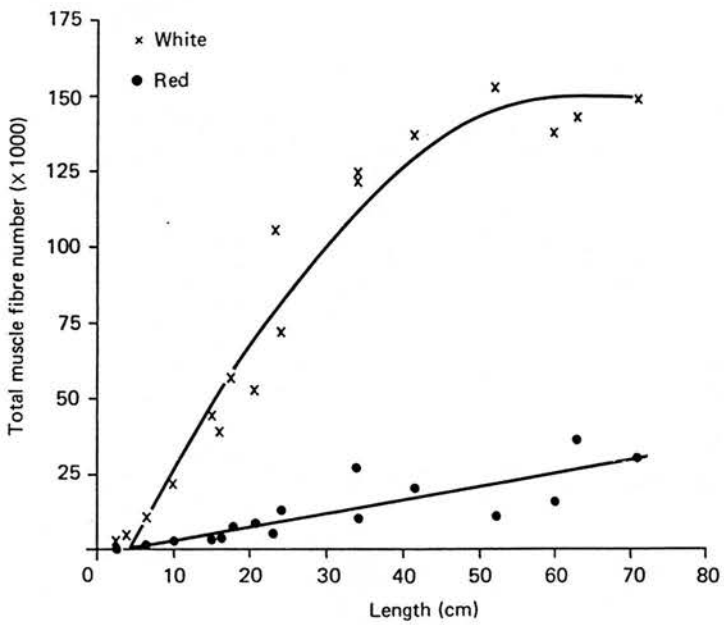

Fig. 2. Total muscle fibre number in complete cross sections for white muscle and red muscle versus fish length.

Table. 1 *Equations of computed regression lines*

Figure	Parameters X	Parameters Y	Line	S_b
1	Length	White muscle CSA	$Y = 0{\cdot}372.X^{2{\cdot}18}$	0·04
	Length	Red muscle CSA	$Y = 0{\cdot}0168.X^{2{\cdot}13}$	0·07
2	Length	Total white fibre no.	$Y = 5505.X - 44.X^2 - 22215$	$S_{b1} = 570$, $S_{b2} = 8{\cdot}0$
	Length	Total red fibre no.	$Y = 434.X - 1055$	74
3	Length	Mean white fibre CSA	$Y = 1383.1{\cdot}041^X$	0·004
	Length	Mean red fibre CSA	$Y = 502.1{\cdot}034^X$	0·004
4	Length	Mean white fibre diameter	$Y = 1{\cdot}64.X + 29{\cdot}5$	0·12
	Length	Mean red fibre diameter	$Y = 24{\cdot}9.1{\cdot}017^X$	0·002
—	White muscle CSA	Total white fibre no.	$Y = 54033.\log X - 49724$	5929
—	White muscle CSA	Mean white fibre CSA	$Y = 5{\cdot}17.X + 1786$	0·22
—	Red muscle CSA	Total red fibre no.	$Y = 10252.\log X + 831$	7489
—	Red muscle CSA	Mean red fibre CSA	$Y = 24{\cdot}6.X + 823$	3·0

CSA, Cross sectional area. S_b, Standard error of regression coefficient. Units: Length, cm; Muscle CSA, mm²; Fibre CSA, μm²; Fibre diameter, μm.

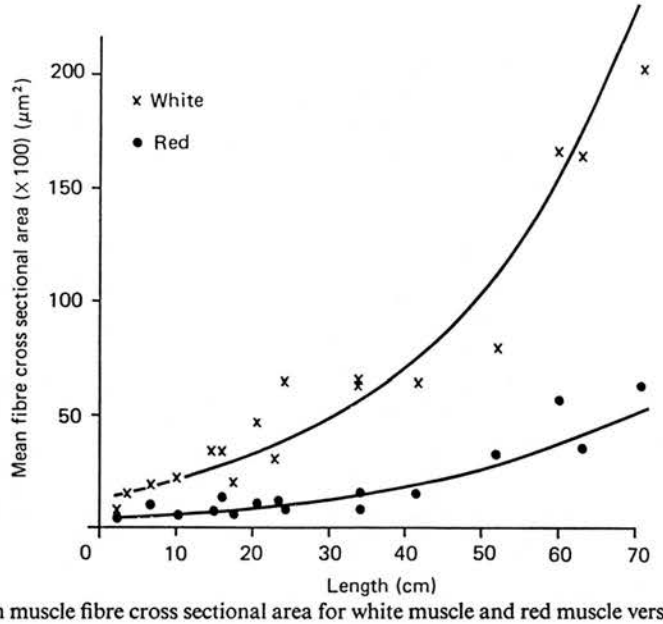

Fig. 3. Mean muscle fibre cross sectional area for white muscle and red muscle versus fish length.

increase in fish length the mean red fibre cross sectional area doubles; the mean white fibre cross sectional area doubles in a slightly shorter time (about every 17 cm). In other words, mean fibre cross sectional area increases at an increasing rate with growth.

Increase in mean white fibre diameter (Fig. 4) shows a linear relationship (Table 1) with fish length, whereas the increase in mean red fibre diameter exhibits an exponential relationship (Table 1) with fish length. From an examination of the 'red' points in Figure 4, however, it would appear that, although the relationship may be

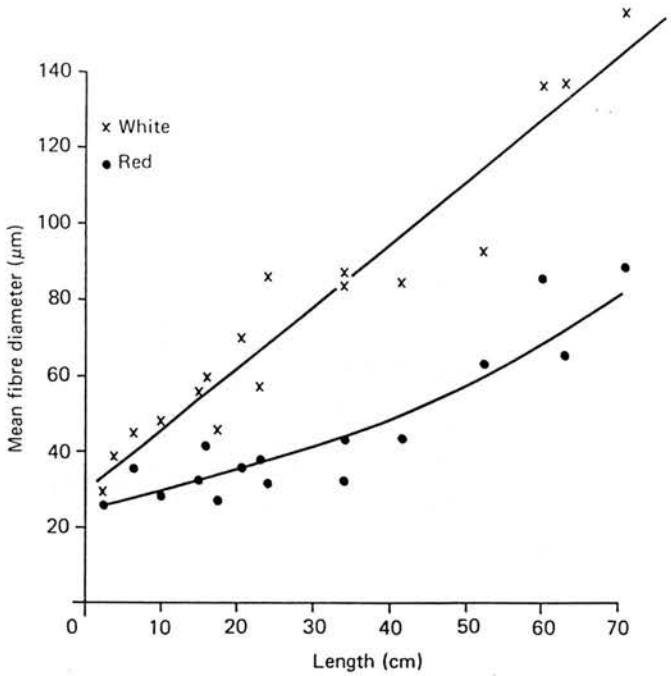

Fig. 4. Mean muscle fibre diameter for white muscle and red muscle versus fish length.

exponential overall (this gave the best-fit regression when using all the points), there is little change in fibre diameter until about 40 cm, which is possibly also shown in Figure 3 for mean red fibre cross sectional area.

Table 1 shows that there is a linear relationship between muscle fibre cross sectional area and total muscle cross sectional area for both red and white muscle, whereas muscle fibre number exhibits a logarithmic relationship with total muscle cross sectional area, also for both white and red muscle. The latter relationship demonstrates that muscle fibre number increase slows down as muscle cross sectional area increases.

Histograms were constructed for muscle fibre diameter distributions for each fish. Figures 5 and 6 show these distributions for white and red muscle, respectively, in representative fish taken at approximately 10 cm intervals. Each white fibre diameter histogram (Fig. 5) is the average for the three white sampled areas (superficial, middle and deep) in each of which 100 fibres were measured. It should be pointed out that within each fish the histograms for the three areas did not show any apparent significant differences and, in particular, did not show evidence of one area having a larger proportion of smaller fibres than another. Figure 5 shows that the increase in mean white fibre diameter during growth is due to an increase in width of the histogram so that, even at 52 cm, there are still some fibres in the smallest size class (10–20 μm) although they represent less than 1 % of the total population at this stage compared to over 10 % at the 2·2 and 10·0 cm stage. In contrast, Figure 6 shows that the increase in mean red fibre diameter is due to the distributions shifting to the right so that, even at 34·0 cm, there are no fibres less than 20 μm in diameter. The distributions are much narrower for the red muscle, especially when it is appreciated that the classes are 5 μm wide in the red (Fig. 6) compared to 10 μm in the white

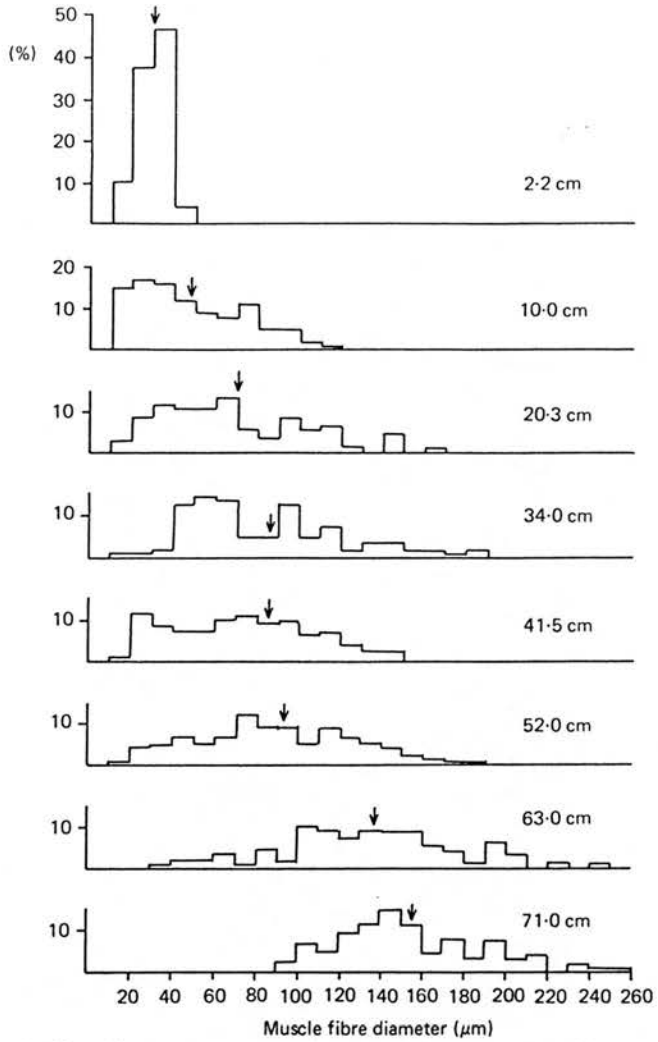

Fig. 5. Muscle fibre diameter percentage frequency histograms for white muscle from fish of lengths indicated alongside each histogram. The arrows mark the mean fibre diameter for each histogram.

(Fig. 5). In fact, for all the histograms shown in Figures 5 and 6, except for the 2·2 cm stage, the variances (based on standard deviations quoted as percentages of the means) are significantly greater ($P < 0{\cdot}05$) for the white muscle than the red.

DISCUSSION

It has been shown that the cross sectional area of both red and white muscle is proportional to approximately the square of fish length (Fig. 1, Table 1), which is perhaps not surprising when plotting an area against a length. Kryvi & Eide (1977), however, found linear relationships between red and white muscle cross sectional areas and fish length in the shark, although this possibly could be explained by the fact that only a relatively narrow range of lengths, namely about 5–25 cm, was used. They also concluded that white muscle cross sectional area increases more than that

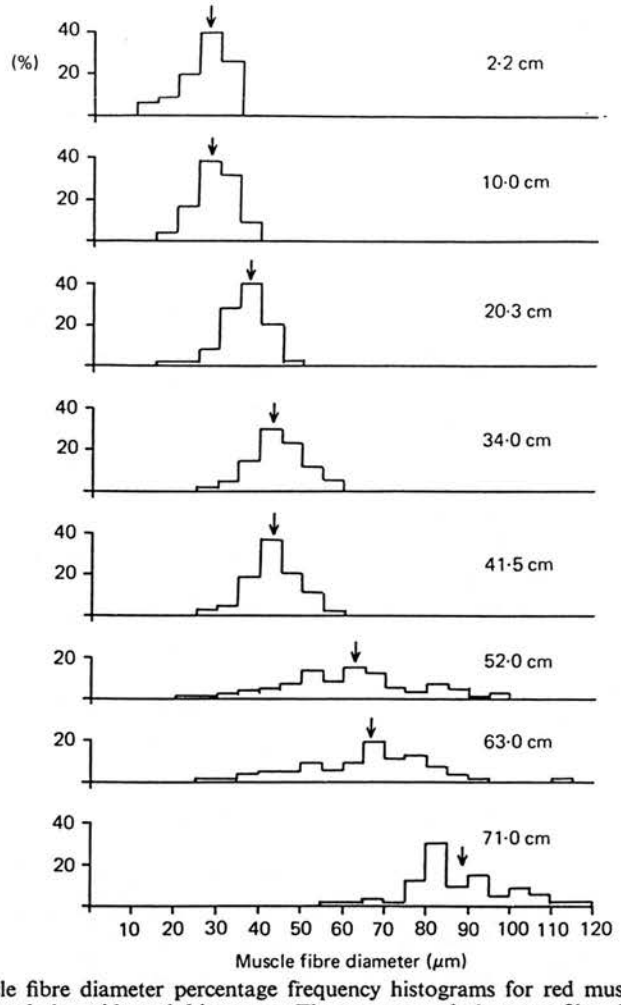

Fig. 6. Muscle fibre diameter percentage frequency histograms for red muscle from fish of lengths indicated alongside each histogram. The arrows mark the mean fibre diameter for each histogram.

of red muscle over the period of study whereas, although the equations in Table 1 show a slightly larger rate of increase in white cross sectional area than that in red, the differences are not significant in this present study.

The rate of increase in the total number of white muscle fibres was found to decrease with increasing fish length (Fig. 2). As far as the red muscle fibres are concerned, there is a definite increase over the length range studied but, owing to the variability found, it is not possible to state with certainty how the rate of increase varies during growth. In contrast to these results, Kryvi & Eide (1977) found no increase in white muscle fibre number in the shark and only a slight increase in that of red. Greer-Walker (1970), however, did find an increase in the number of red and white muscle fibres (added together) throughout the growth of cod from about 5 to 120 cm, but with no rate decrease at the upper end of the range.

It is possible that any change in muscle fibre number with growth may be due to a change in the arrangement of muscle fibres within the muscle. An apparent increase in fibre number may be due to the growth of intrafascicularly-terminating fibres

entering the plane of section. However, the lateralis muscle of fish is segmented into myomeres which are separated by sheets of connective tissue, the myocommata. Within these myomeres the muscle fibres are only a few millimetres long and run, roughly parallel to the long axis of the body, from one myocomma to the next. No fibre terminations have been observed between the myocommata when muscle samples have been teased under a microscope (personal observation). It is also known that, although individual fibres extend only from one myocomma to the next, successive fibres lying on opposite sides of myocommata can be teased out as long threads over many myomeres; these threads are known as muscle fibre trajectories (Alexander, 1969). These observations, together with the fact that the arrangement and number of myomeres do not change with growth, indicate that any increase observed in the number of muscle fibres per cross section must be due to the formation of new muscle fibres.

As far as muscle fibre diameter is concerned, there is a continuing increase when plotted against length (Fig. 4) which appears exponential overall for the red muscle although linear for the white. In his study on the cod, however, Greer-Walker (1970) found that red and white muscle fibre diameters increased in a sigmoid manner which, in the white muscle, was followed by a decrease from 83 cm onwards.

The relative contributions of hyperplasia and hypertrophy to increase in muscle cross sectional area were calculated for both white and red muscle and the results are shown in Figure 7. This figure was calculated by estimating the increase in fibre number per unit length (every 5 cm) from Figure 2, and the increase in fibre cross sectional area per unit length from Figure 3, and expressing one increase as a percentage of the two combined. This figure shows that, for the white muscle (Fig. 7*a*), hyperplasia is at first the major contribution to the increase in muscle cross sectional area but throughout growth its relative importance decreases so that at the end of the trout's growth (65–70 cm) increase in muscle cross sectional area is due entirely to fibre hypertrophy. Based on biochemical analyses of rainbow trout muscle (DNA content and ratio of fresh weight to DNA), Luquet & Durand (1970) concluded that hyperplasia accounted for 70 % of the increase in muscle weight up to 400 g body weight (about 33 cm) and thereafter continued to account for 90 %, cells having reached 75 % of their final size by 400 g. It must be appreciated, however, that the hyperplasia to which the authors referred was increase in the number of nuclei, not fibre hyperplasia, and their conclusions on cell size indicate only that the cytoplasmic to nuclear ratio shows little increase after 400 g.

It is interesting to note from Figure 5 that the first histogram in which there are no fibres in the smallest size class (10–20 μm) is that for the 63 cm fish. It may be reasonable to assume that a lack of these very small fibres indicates that no new fibres are being formed, i.e. that hyperplasia has ceased. If this is true then the histogram results are in agreement with Figure 7*a* which shows that hyperplasia does, in fact, cease by this 63 cm stage. Weatherley *et al.* (1979) produced histograms for fibre diameters in the white muscle of rainbow trout up to about 33 cm in length. They did not count the number of fibres but deduced, in agreement with this present investigation, that, as the number of fibres in the smallest size class was reducing, so hypertrophy became relatively more important than hyperplasia in muscle growth.

The large range of fibre diameters seen in the white muscle of trout give it a characteristic mosaic appearance (Boddeke *et al.* 1959) which was once assumed to be a mixture of small red and larger white fibres. Johnston *et al.* (1975), however, showed that both the small and large fibres of this mosaic muscle had the same

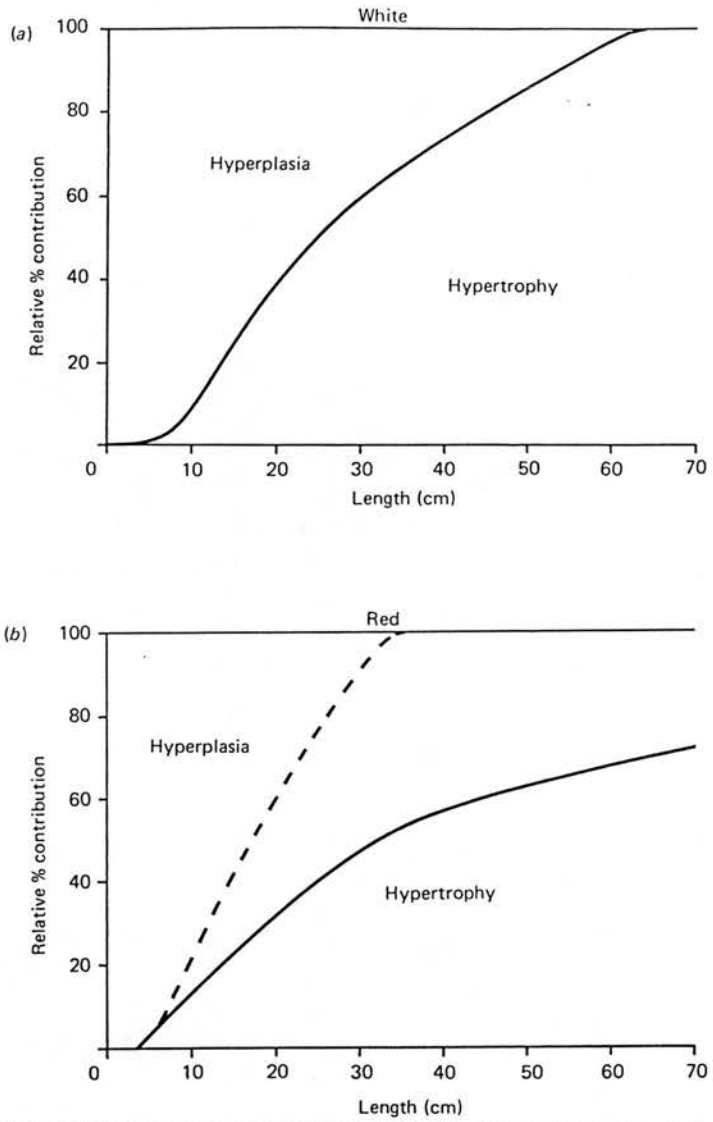

Fig. 7 (*a–b*). Relative percentage contributions of hyperplasia and hypertrophy to total muscle cross sectional area growth versus fish length for (*a*) white muscle and (*b*) red muscle. The broken line in (*b*) is explained in the text.

histochemical properties. Carpenè & Veggetti (1981) working on Mugilidae fish species found small fibres in the white muscle with different histochemical properties from the rest of the white muscle. They concluded that small fibres were produced at certain times of the year and enlarged to evolve mature white fibre properties. It seems reasonable to assume here, therefore, that the smallest fibres are the newly formed fibres which gradually enlarge, hence the widening histograms in Figure 5. It is possible that the seasonal variation observed by Carpenè & Veggetti (1981) may be present in the rainbow trout. For example, in Figure 4, the fish at 24 cm has, in relation to its very small length difference, much bigger white muscle fibres than the fish at 23 cm. The former was obtained in November whereas the latter was obtained in June. The data from this investigation are insufficient, however, to indicate whether

this seasonal variation is significant in the trout; more work needs to be carried out in order to elucidate this point. On the other hand, a difference in fibre diameters between fish at the same length may be due to these fish having different growth rates with the fastest growing fish having smaller fibres (Weatherley *et al.* 1979).

As mentioned earlier the red muscle situation is difficult to assess. The continuous line in Figure 7*b* is based on the 'red' lines in Figures 2 and 3. If a disappearance of fibres in the smallest size class is an indication of the cessation of hyperplasia, then, from Figure 6, this occurs at 34 cm (the first stage when there are no fibres less than 20 μm). This would then support the validity of the broken line shown in Figure 7*b*, so that from 34 cm onwards all increase in red muscle cross sectional area is due to fibre hypertrophy. However, although it is perhaps most likely that hyperplasia does cease by about 34 cm, the variability in the results (particularly those shown in Figure 2 'red') means that the possibility that hyperplasia continues throughout growth cannot be ruled out, althouth its relative contribution must decrease.

In conclusion, it has been shown that postlarval muscle growth in the trout is due, firstly and mainly, to hyperplasia, which is then gradually replaced by hypertrophy as the major factor until hyperplasia ceases completely. Although this is somewhat analogous to the prenatal growth of mammalian muscle (Stickland, 1981), there is little hyperplasia postnatally in mammalian muscle (Goldspink, 1962). The newly formed fibres found in growing fish muscle are probably produced from satellite cells or persistent myoblasts rather than by fibre splitting. No evidence of fibre splitting has been observed in the samples used in this investigation. The exact mechanism of fibre production is, however, being presently examined in this laboratory. Another interesting question posed by the results described here is how the new muscle fibres became innervated. Easter (1979) found that there was no increase in axon number in the trochlear nerve supplying the superior oblique eye muscle in the goldfish during growth, although the number of neuromuscular synapses increased greatly to accommodate the more numerous muscle fibres. The innervation of the newly formed fibres found in the lateralis muscle is presently being investigated in this laboratory.

SUMMARY

The growth of red and white muscle was investigated in the rainbow trout, using fish from 2·2 cm to 71 cm in length. In the white muscle, fibre hyperplasia, initially, accounted for all muscle growth but its relative contribution decreased as the contribution from fibre hypertrophy increased. At and above about 65 cm there was no hyperplasia, and this corresponded approximately to the stage when there were no more fibres in the smallest size class (less than 20 μm). The results for the red muscle are more variable and hence more difficult to assess. Although red fibre hyperplasia may continue throughout growth, at 34 cm there are no fibres in the smallest size class, which possibly indicates no new fibre formation beyond this stage.

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REFERENCES

ALEXANDER, R. M. (1969). The orientation of muscle fibres in the myomeres of fishes. *Journal of the Marine Biological Association, U.K.* **49**, 263–290.

BODDEKE, R., SLIJPER, E. J. & VAN DER STELT, A. (1959). Histological characteristics of the body musculature of fishes in connection with their mode of life. *Koninklijke Nederlandse Akademie van Wetenschappen–Amsterdam, Proceedings, Series C* **62**, 576–588.

CARPENÈ, E. & VEGGETTI, A. (1981). Increase in muscle fibres in the lateralis muscle (white portion) of Mugilidae (Pisces, Teleostei). *Experientia* **37**, 191–193.

EASTER, S. S. (1979). The growth and development of the superior oblique muscle and trochlear nerve in juvenile and adult goldfish. *Anatomical Record* **195**, 683–697.

GOLDSPINK, G. (1962). Studies on postembryonic growth and development of skeletal muscle. *Proceedings of the Royal Irish Academy* B **62**, 135–150.

GREER-WALKER, M. (1970). Growth and development of the skeletal muscle fibres of the cod (*Gadus morhua* L.). *Journal du Conseil – conseil permanent international pour l'exploration de la mer* **33**, 228–244.

JOHNSTON, I. A., WARD, P. S. & GOLDSPINK, G. (1975). Studies on the swimming musculature of the rainbow trout. I. Fibre types. *Journal of Fish Biology* **7**, 451–458.

KRYVI, H. & EIDE, A. (1977). Morphometric and autoradiographic studies on the muscle fibres in the shark *Etmopterus spinax*. *Anatomy and Embryology* **151**, 17–28.

LUQUET, P. & DURAND, C. T. (1970). Évolution de la teneur en acides nucléiques de la musculature épaxiale au cours de la croissance chez la truite arc-en-ciel (*Salmo gairdneri*); rôles respectifs de la multiplication et du grandissement cellulaires. *Annales de biologie animale, biochimie et biophysique* **10**, 481–492.

RAYNE, J. & CRAWFORD, G. N. C. (1975). Increase in fibre numbers of the rat pterygoid muscles during postnatal growth. *Journal of Anatomy* **119**, 347–357.

ROWE, R. W. D. & GOLDSPINK, G. (1969). Muscle fibre growth in five different muscles in both sexes of mice. I. Normal mice. *Journal of Anatomy* **104**, 519–530.

STICKLAND, N. C. (1981). Muscle development in the human fetus as exemplified by m. sartorius: a quantitative study. *Journal of Anatomy* **132**, 557–579.

WEATHERLEY, A. H., GILL, H. S. & ROGERS, S. C. (1979). Growth dynamics of muscle fibres, dry weight, and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). *Canadian Journal of Zoology* **57**, 2385–2392.

WEATHERLEY, A. H., GILL, H. S. & ROGERS, S. C. (1980). Growth dynamics of mosaic muscle fibres in fingerling rainbow trout (*Salmo gairdneri*) in relation to somatic growth rate. *Canadian Journal of Zoology* **58**, 1535–1541.

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Anatomy and Embryology

The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L.)

N.C. Stickland[1], R.N. White[1], P.E. Mescall[1], A.R. Crook[1], and J.E. Thorpe[2]

[1] Department of Anatomy, The Royal Veterinary College, Royal College Street, London NW1 OTU, United Kingdom
[2] Freshwater Fisheries Laboratory, Pitlochry. Perthshire PH16 5LB, United Kingdom

Summary. From fertilisation to hatching one group of salmon embryos was reared at ambient temperatures (fluctuating around 1.6° C) and another at 10° C. At Gorodilov stages 28, 30 and 33 transverse sections of whole embryos were obtained for light and electron microscopy. Total cross-sectional areas, fibre numbers, fibre diameters and myofibrillar areas of the white muscle of m. lateralis were measured. At hatching (stage 33, which occurred much earlier at the higher temperature), the higher temperature embryos had significantly larger ($P<0.01$) but fewer ($P<0.05$) muscle fibres. These larger fibres contained significantly more myofibrillar material ($P<0.05$) than the smaller fibres of the lower temperature embryos. Lesser differences were found at pre-hatching stages. Higher temperatures caused myofibre hypertrophy to increase at a greater rate than hyperplasia. Hence, the cellularity of the tissue produced under the different temperature regimes was quite different.

Key words: Salmon – Myogenesis – Temperature

Introduction

It has long been known (Krogh 1914) that the rate of embryonic development is influenced by temperature in a variety of animals, and in the salmon this was demonstrated by Hayes et al. (1953). However, less is known about the effects of temperature on cellular aspects of developing tissues. Embryonic stages of myogenesis in fish are associated with myofibre hyperplasia as well as hypertrophy. The present study investigated the effects of temperature on these two features of embryonic muscle development in Atlantic salmon (*Salmo salar* L.).

Materials and methods

Eggs were stripped from a 4-year-old female salmon on 28 November 1985, and fertilised with sperm from a 4-year-old male. They were transferred to perspex hatching trays in mesh baskets, which were kept in hatching troughs through which water flowed at 1.5–2.0 l/min, at the DAFS Smolt Rearing Station, Almondbank, Perthshire. Half the eggs were incubated at 10° C, maintained constant by means of a proportional electronic thermostat. The other half were kept at ambient temperature which fluctuated around a daily median of 1.6° C (S.E. 0.2) for the study period (127 days from fertilisation).

From the onset of somitogenesis, samples of 10 eggs from each temperature regime were sent by train to London at defined intervals, wrapped in cotton wool soaked in river water, inside a jar cooled with ice. All eggs arrived alive.

Development stages were defined according to Gorodilov's (1983) system. Stages 28, 30 and 33 were used, corresponding to ages 83, 103, and 127, and 34, 40 and 54 days after fertilisation for the ambient and 10° C embryos respectively. For both regimes, stage 33 were newly hatched.

The embryos were removed from their shells, decapitated and the first 15-somite region placed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (at pH 7.3) for 2–3 h. Then they were post-fixed in 1% osmium tetroxide, dehydrated in acetones and embedded in araldite. Semi-thin (0.5–1 μm) transverse sections were cut and stained with methylene blue. These complete body-sections, taken between the 10th and 15th somites, were used for the light microscopic analyses. Ultrathin sections were obtained from stage 33 for electron microscopy.

A total of 18 embryos (3 at each stage, under each regime) were analysed. The whole transverse sectional area of muscle (m. lateralis) on one side was measured using a VIDS II image analyser (Analytical Measuring Systems Ltd). The number of fibres in this area was counted from photomicrographs (Fig. 1). Superficial red muscle was excluded from all analyses. Cell size analysis was made from a region dorsal to the lateral line, excluding the most superficial white fibres (Fig. 1). As it was impossible to identify fibre boundaries in the light microscope sections, mean myofibrillar areas (readily seen within individual fibres) were measured in up to 100 fibres in each region. To assess variation in these areas across the muscle blocks, myofibrillar areas were measured across a transect for all the stage 33 fish (Fig. 1).

Electron micrographs (from the square sample area, Fig. 1) of 10 fibres were analysed by the VIDS system for all stage 33 fish, to assess the relationship between muscle fibre area and myofibrillar area for each temperature regime. The significances of differences in various parameters were determined by Student's *t*-test between temperature regimes.

Results

The salmon embryos developed much faster at the higher temperature (Fig. 2).

Offprint requests to: N.C. Stickland

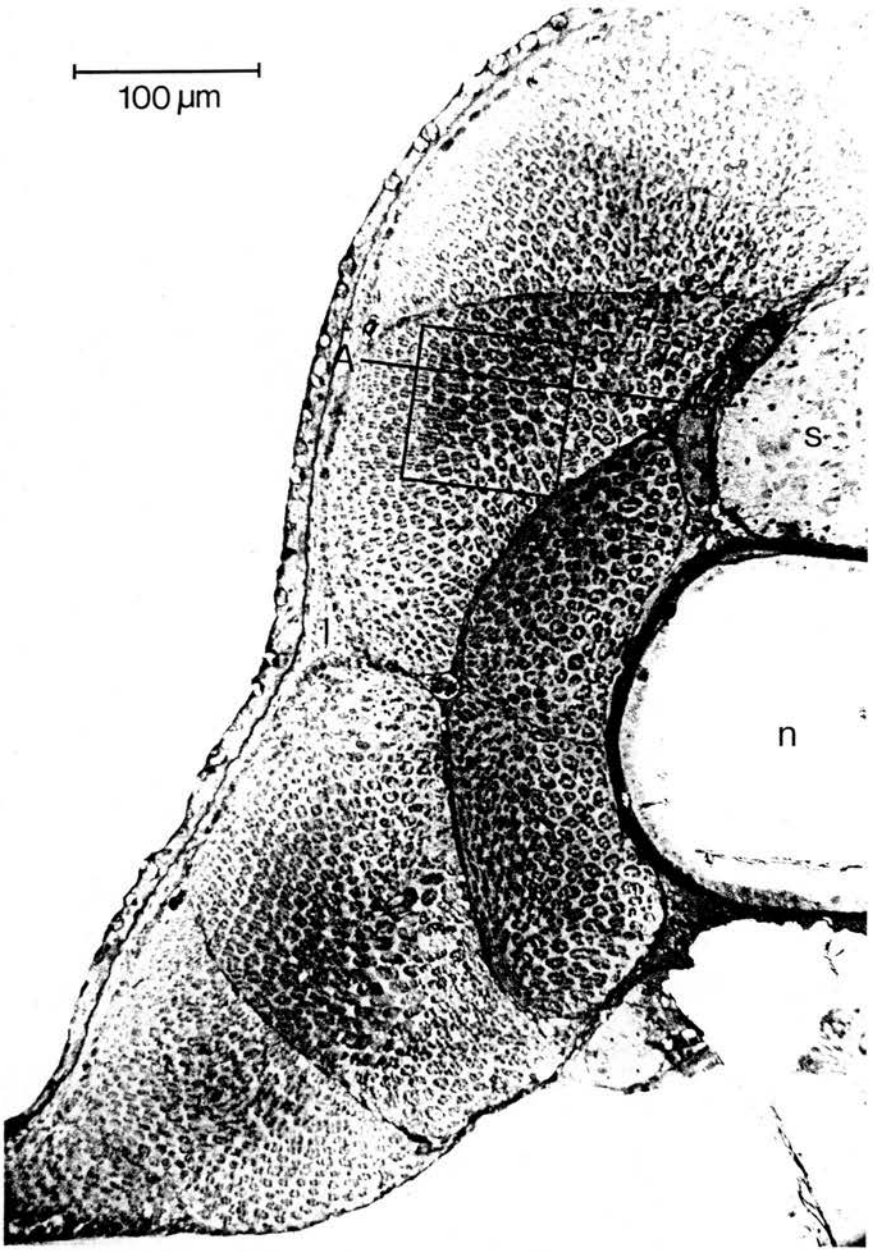


Fig. 1. A complete transverse section of one side of a newly hatched fish reared at ambient temperature. The line *AB* indicates the position of a typical transect along which all myofibrillar areas were measured. The box indicates the region where measurements were made to estimate mean muscle fibre cross-sectional areas and myofibrillar areas. *l*, lateral line; *s*, spinal cord; *n*, notochord

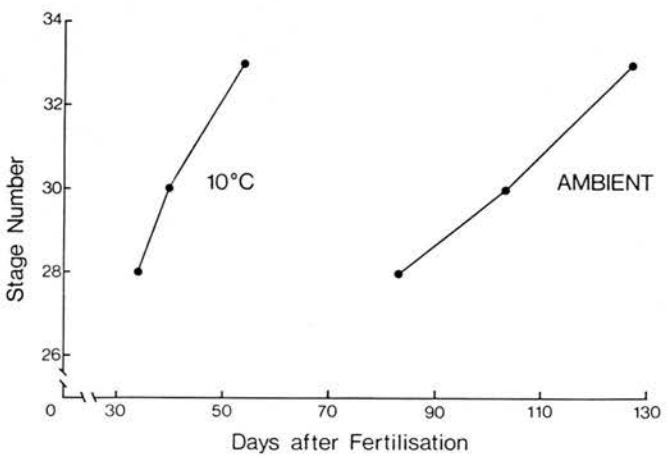


Fig. 2. Shows the relationship between stage number and days after fertilisation for each temperature regime

The total muscle cross-sectional area (of one side) of the ambient embryos was slightly but not significantly larger than the 10° C embryos at all stages (Fig. 3). Also, there was no significant difference between the lengths of ten ambient and ten 10° C embryos at stage 33. At stages 28 and 30, there were no significant differences between treatments in the total number of muscle fibres in the m. lateralis of one side, but these increased at a much greater rate with respect to stage number in the ambient embryos (Fig. 4) so that at hatching the 10° C embryos contained about 30% fewer fibres. Mean myofibrillar areas were significantly larger in the 10° C embryos at stages 28 and 33 (80% and 50% larger respectively) (Fig. 5).

Myofibrillar areas from a transect (Fig. 1) of representative ambient and 10° C embryos at stage 33 gradually increased in size from the lateral towards the middle region of the muscle (Fig. 6). The size of these areas then levelled off before falling rapidly at the most medial border. The

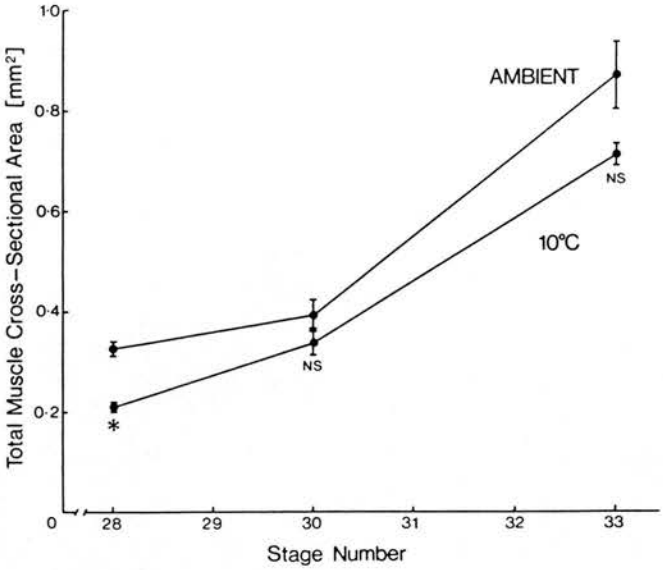


Fig. 3. Shows the relationship between total muscle cross-sectional area (for m. lateralis on one side) and stage number for each temperature regime. Significance of differences at each stage are indicated: *NS*, Non-significant; *, $P<0.02$

larger size and fewer number of fibres was also evident in the 10° C embryos.

Electron micrograph measurements on the stage 33 embryos showed that myofibrils occupied significantly more ($P<0.05$) of the fibre areas in the 10° C embryos (64.8% ± 3.9) than in the ambient ones (59.3% ± 1.5) (Fig. 7).

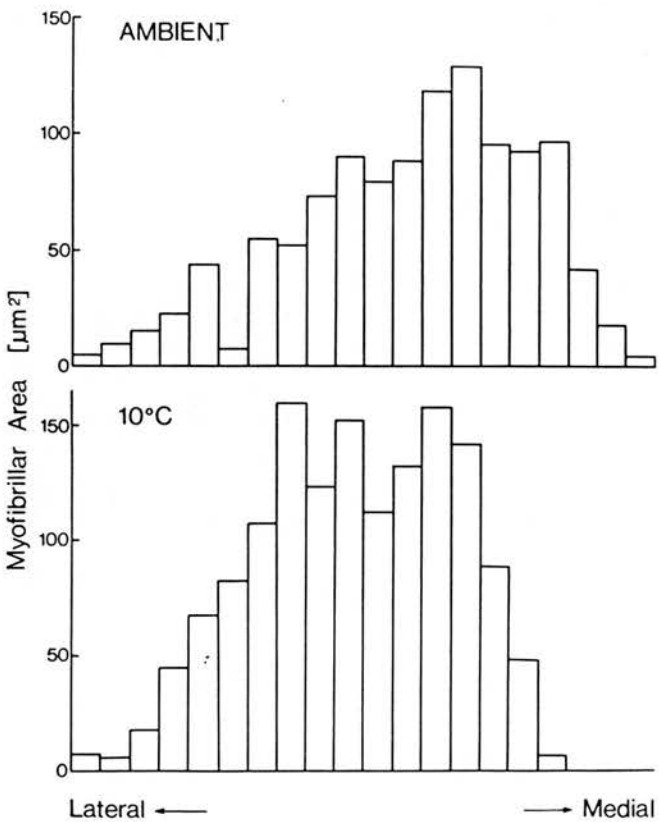
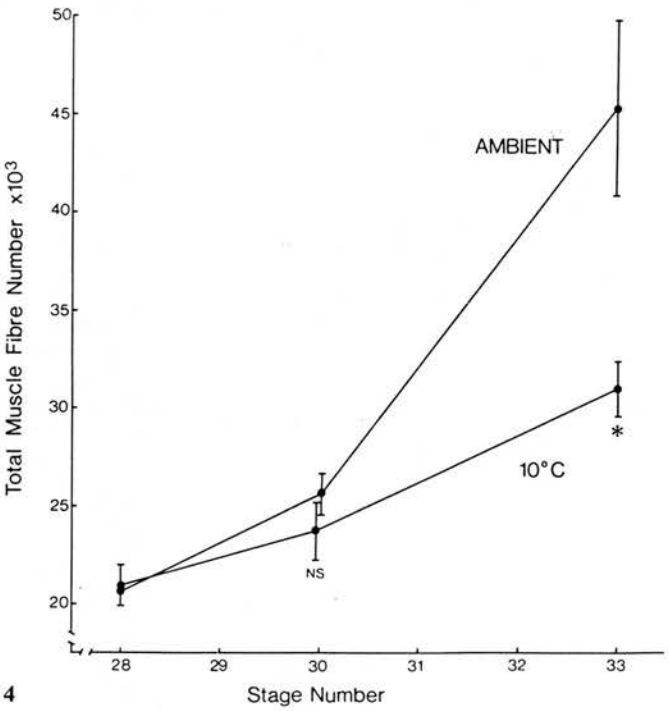


Fig. 6. Shows the change in myofibrillar area across m. lateralis (of one side) from the most lateral to most medial aspect



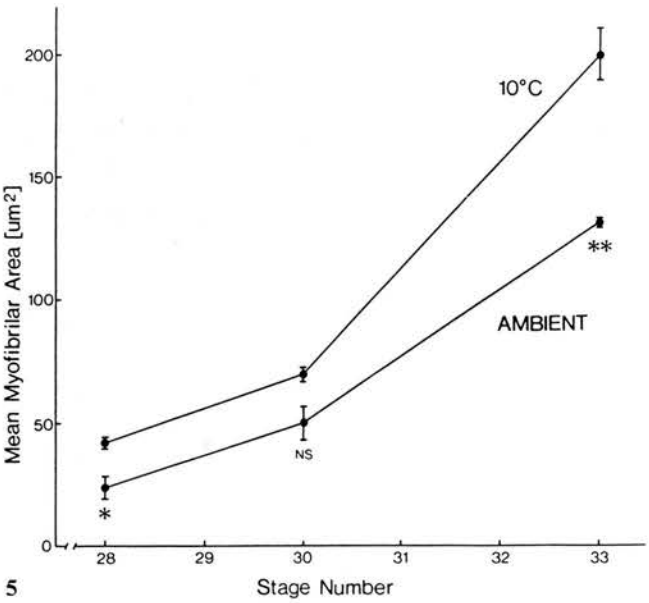
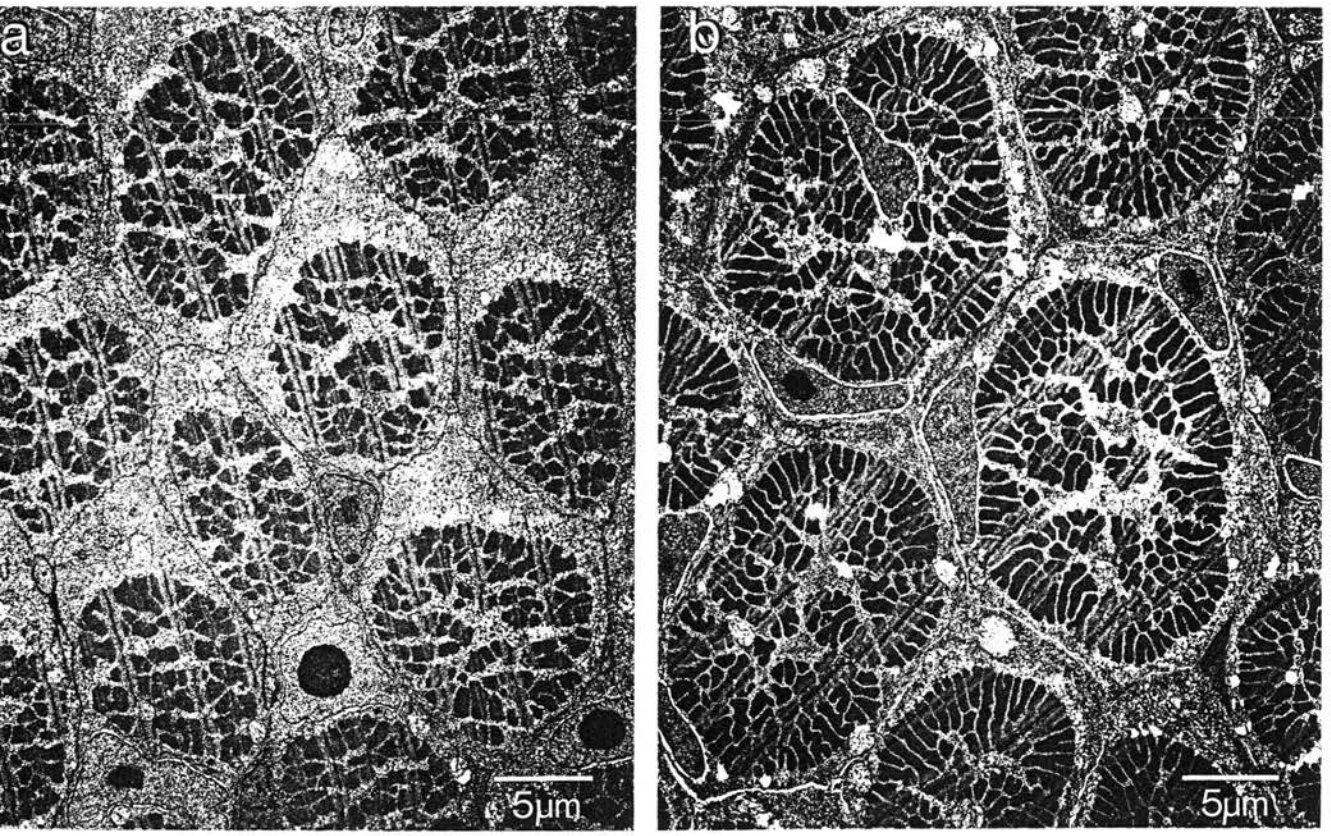


Fig. 4. Shows the relationship between total muscle fibre number (in m. lateralis of one side) and stage number for each temperature regime. Significance of differences at each stage are indicated: *NS*, Non-significant; *, $P<0.05$

Fig. 5. Shows the relationship between mean myofibrillar area and stage number for each temperature regime. Significance of differences at each stage are indicated: *NS*, Non-significant; *, $P<0.05$; **, $P<0.01$



. 7. Electron micrographs of muscle taken from newly hatched fish reared at (**a**) ambient temperature and (**b**) 10° C

scussion

gher rearing temperatures increase the developmental e of fish embryos and advance the time of hatching ayes et al. 1953; Kinne and Kinne 1962; Balon 1980), d the present study confirms this. It is also clear that developmental rate of all the muscle parameters mea- ed has been increased by the higher rearing temperature. owever, to assess differential effects of temperature on scle parameter development the embryos have to be mpared at the same developmental stage. Hayes et al. 53) suggested that the order of appearance of anatomical tures in salmon morphogenesis might be altered by tem- rature, but Gorodilov (1983) showed a constancy in this uence independent of temperature between 0.5 and C. We therefore used developmental stages as defined Gorodilov as the instants for quantitative comparison myogenesis under the contrasted temperature regimes.

Both fibre hyperplasia and hypertrophy occurred during mon myogenesis in this study. Hyperplasia was evi- ced by an increase with age in the total number of myo- es seen in the complete body sections. Hypertrophy was n as an increase in both fibre cross-sectional area and myofibrillar content. Both fibre hyperplasia and hyper- phy continue in post-hatch salmonid muscle growth eatherley et al. 1979, 1980; Stickland 1983; Villarreal 3; Higgins 1985), whereas in mammals hyperplasia ses near the time of birth (Stickland 1981; Wigmore Stickland 1983). If it is accepted that the newest fibres the smallest, then the transect histograms (Fig. 6) indi- e that new fibres form at the lateral borders of the myo- ne. Waterman (1969) found a similar situation in *Brachy-*

danio rerio. There also appear to be zones of proliferation dorsally and ventrally in the salmon myotome (Fig. 1). In post-hatch salmon this lateral proliferation zone is not evident, and very small fibres are found throughout the myotome (Brooks, personal communication).

The most interesting result is the finding that temperature had a greater effect on the rate of embryonic fibre hypertrophy (as measured by myofibrillar area increase) than on hyperplasia. It would seem that temperature had a differential effect on cell division and protein synthesis. By the time of hatching the higher temperature embryos had fewer but 40% larger muscle fibres than the ambient embryos. Also, as their myofibrillar areas were 50% greater, the 10° C embryos had relatively more myofibrillar material in their fibres at hatching ($P<0.01$). This may be due to increased temperatures causing increased embryonic movements (Fluchter and Rosenthal 1965), in turn stimulating myofibril production. However, this contrasts with the general finding that fibre hyperplasia is greater among faster than among slower growing fishes (Weatherley and Gill 1987), and requires further study.

In summary, higher incubation temperature produced muscles at hatching with fewer but larger fibres, with relatively higher myofibrillar content. So although higher temperatures increased the development rate, not all mechanisms of myogenesis were increased to the same extent. Hence the cellularity of the tissue produced differed under different temperature regimes.

Acknowledgements. We thank M.S. Miles and his staff at the Almondbank Smolt Rearing Station for careful husbandry of the salmon, and for arranging the transport of the eggs.

References

Balon EK (1980) Charrs: Salmonid fish of the genus *Salvelinus*. Junk Publishers, The Hague

Fluchter H, Rosenthal P (1965) Beobachtungen über das Vorkommen und Leichen des blauen Wittlings (*Micromesistius poutassou* Risso) in der deutschen Bucht. Helgol Wiss Meeresunders 12:149–155

Gorodilov Yu N (1983) Stadii embryonalnogo razvitiya atlanticheskogo lososya *Salmo salar* L. II. Opisaniye i khronologia. Sb Nauchn Tr GosNIORKh 200:107–126 Transl Thorpe JE, Scott Fish Res Transl 19:1–34

Hayes FR, Pelluet D, Gorham E (1953) Some effects of temperature on the embryonic development of the salmon (*Salmo salar*). Can J Zool 31:42–51

Higgins PJ (1985) Metabolic differences between Atlantic salmon (*Salmo salar*) parr and smolts. Aquaculture 45:33–53

Krogh A (1914) On the influence of the temperature on the rate of embryonic development. Allg Physiol 16:163–177

Kinne O, Kinne EM (1962) Rates of development in embryos of a cyprinodont fish exposed to different temperature-salinity-oxygen combinations. Can J Zool 40:231–253

Stickland NC (1981) Muscle development in the human fetus as exemplified by m. sartorius: a quantitative study. J Anat 132:557–579

Stickland NC (1983) Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*). J Anat 137:323–333

Villarreal CA (1983) The role of light and endocrine factors in the development of bimodality of growth in the juvenile Atlantic salmon (*Salmo salar* L.). PhD Thesis, University of Stirling, p 381

Waterman RE (1969) Development of the lateral musculature in the teleost, *Brachydanio rerio*: a fine structural study. Am J Anat 125:457–492

Weatherley AH, Gill HS (1987) The biology of fish growth. Academic Press, London, p 443

Weatherley AH, Gill HS, Rogers SC (1979) Growth dynamics of muscle fibres, dry weight and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). Can J Zool 57:2385–2392

Weatherley AH, Gill HS, Rogers SC (1980) Growth dynamics of mosaic muscle fibres in fingerling rainbow trout (*Salmo gairdneri*) in relation to somatic growth rate. Can J Zool 58:1535–1541

Wigmore PMC, Stickland NC (1983) Muscle development in large and small pig fetuses. J Anat 137:235–245

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Muscle development in Atlantic salmon (*Salmo salar*) embryos and the effect of temperature on muscle cellularity

M. L. USHER*, N. C. STICKLAND* AND J. E. THORPE†

**Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU and †SOAFD Freshwater Fisheries Laboratory, Pitlochry, Perthshire PH16 5LB, U.K.*

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Salmon eggs were incubated at 5, 8 or 11° C from fertilization to hatching. At Gorodilov stages 25, 27, 29, 31 and 33 transverse sections of whole embryos (at somite level 10–15) were prepared for histochemistry and electron microscopy. At every stage up to hatching, cross-sectional areas of the embryos were not different between temperatures, and from stage 27 onwards there was also no difference in the ratio of white to red muscle. However, there were more muscle fibres but of smaller average diameter in both the red and white muscle for the colder temperature embryos. At hatching there were also more nuclei (per cross-section) in the colder embryos but more nuclei per muscle fibre in the warmer embryos. In all cases the 8° C embryos were intermediate between 5 and 11° C embryos in their muscle parameters. Fast and slow muscle fibres could only be distinguished in the embryos by alkali-stable ATPase reactions. Succinic dehydrogenase activity was low in embryonic fish. No differences between the temperature groups were detected in the histochemical reactions for either ATPase or succinic dehydrogenase activities.

Key words: *Salmo salar*; skeletal muscle; temperature; development.

I. INTRODUCTION

The rate of embryonic development has long been known to be influenced by temperature in a variety of animal species (Krogh, 1914). This effect was demonstrated in the salmon (*Salmo salar*, L.) by Hayes *et al.* (1953). In a previous communication (Stickland *et al.*, 1988) it was shown that the rearing of salmon embryos at ambient temperatures (fluctuating around 1·6° C) and 10° C produced a very marked difference in the cellularity of the muscle. At hatching, which occurred much earlier in the 10° C embryos, the higher temperature embryos had significantly larger but fewer muscle fibres. Lesser differences were found at two earlier stages of development. It would seem that temperature had a greater effect on the rate of embryonic fibre hypertrophy than on hyperplasia. This contrasts with the general finding in post-hatch fish that fibre hyperplasia is greater among faster than among slower growing fish (Weatherley & Gill, 1987; Higgins & Thorpe, 1990). The salmon embryos also contrast with the findings of Vieira & Johnston (1992) who showed that higher rearing temperatures for herring embryos (*Clupea harengus*, L.) produced more, but smaller muscle fibres. The apparent anomaly of the salmon embryos clearly requires further study.

The aims of the present investigation were to extend the studies of Stickland *et al.* (1988) by examining more embryonic stages and at less extreme temperatures. 10° C is nearer the upper tolerable limit for salmon embryos and may therefore not represent the situation for smaller temperature differences. It

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might be suggested that the inability to produce more muscle fibres at higher temperatures may be a result of relatively low rates of nuclear proliferation. Estimates of nuclear numbers were therefore made in this present study.

Many studies in post-larval fish have shown that cold temperatures increase the amount of red muscle tissue relative to white (Johnston & Lucking, 1978). Another aim of this present study was to determine the cellularity and relative proportion of red to white muscle at different temperatures. Furthermore, the opportunity was also taken to investigate the histochemical differentiation of muscle tissue in the salmon which to date has only been studied in the postlarval stages (Higgins, 1990).

II. MATERIALS AND METHODS

EMBRYOS

Three groups of salmon eggs (500) derived from a single adult pairing were incubated separately at 5, 8 and 11° C from fertilization to hatching. The eggs were maintained at the SOAFD smolt rearing station, Almondbank, Scotland. Each group of embryos was sampled at intervals corresponding to Gorodilov (1983) stages 25, 27, 29, 31 and 33 (hatching).

TISSUE PREPARATION

Ten embryos per stage were prepared as described previously (Stickland *et al.*, 1988), for electron microscopy. Semi-thin sections (0·35–1·0 μm) in the region of somites 10–15 were stained according to Ontell (1974). This proved to be a better method than routine methylene blue for distinguishing individual muscle fibres. To ensure adequate staining intensity it was found necessary to pre-treat sections with ethanolic KOH for 15 min (Jones, 1979). Preliminary studies showed that, whereas the most caudal somites were less developed than cranial somites, there were no significant differences within somites 10 to 15 for the stages under investigation.

MUSCLE MEASUREMENTS

All measurements on the embryo sections were made using a Seescan Image Analyser linked to an Olympus research microscope. The cross-sectional area of red muscle and of white muscle from both sides was measured (Fig. 1) and the total number of muscle fibres contained within those areas was counted. The total number of muscle nuclei within the white muscle area was also counted. At high magnification it was possible to eliminate non-muscle nuclei including all those within the myosepta. Muscle nuclei might, however, include any satellite cell nuclei which might be present. It was hoped that the Ontell (1974) technique would distinguish satellite cell nuclei but, either the technique did not work well, or negligible numbers of satellite cells were present. Mean myofibre areas and mean myofibrillar areas (based on 100 fibres per sample) were also estimated for the white muscles in a smaller rectangular area indicated in Fig. 1. The special staining technique described above ensured that myofibrils could be readily distinguished under high magnification. Differences between temperature treatments for any given stage were assessed by analysis of variance followed by a Neuman–Keul test with a probability of less than 5% being deemed significant. All mean measurements were based on at least five fish per stage per temperature treatment and, for each fish, six sections were analysed.

MUSCLE HISTOCHEMISTRY

For each sampled stage and temperature some larvae were rapidly frozen, supported by mounting medium, in dichlorodifluoromethane (Arcton 12, ICI Ltd) cooled to its

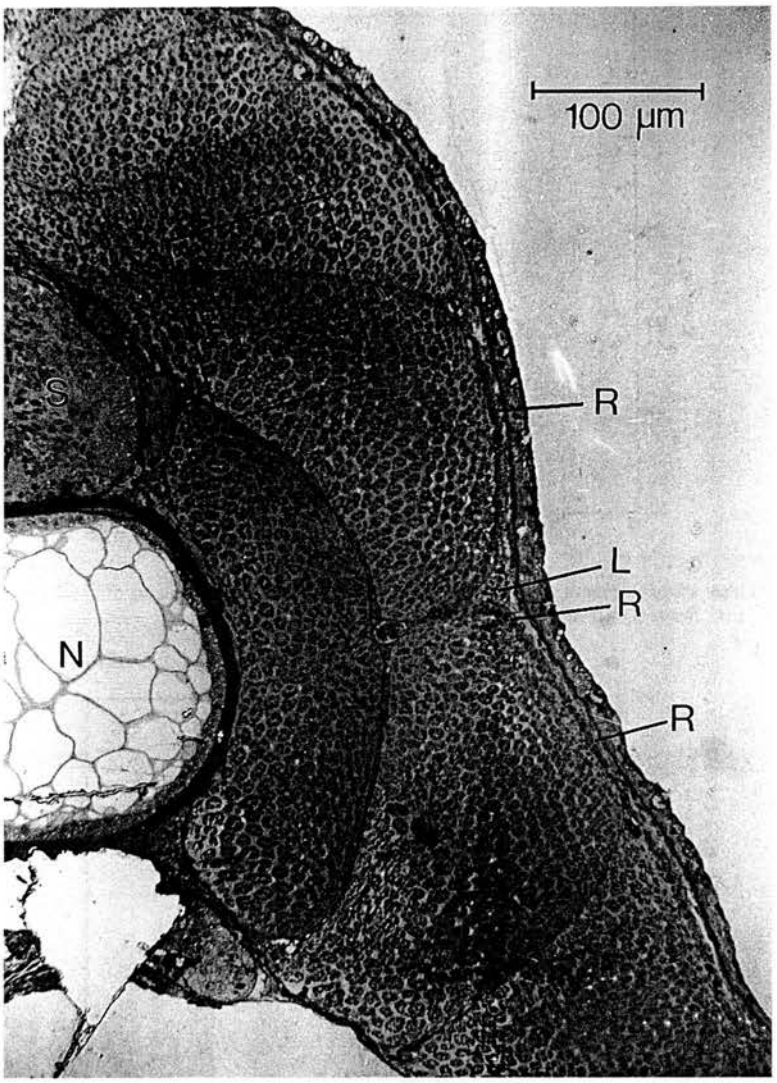

FIG. 1. Transverse section of part of one side of a newly hatched (stage 33) fish. Muscle fibres and nuclei were counted in the whole section (both sides). The box indicates the site of white muscle fibre area and white muscle myofibrillar area measurements. R, Red muscle; S, spinal cord; N, notochord; L, lateral line.

melting point of −158°C in liquid nitrogen. Ten-µm frozen sections were cut on a cryostat.

Succinic dehydrogenase (SDH) activity was measured by the method of Nachlas *et al.* (1957). Myofibrillar ATPase activity was measured based on the Johnston *et al.* (1974) modification of Guth & Samaha's (1970) method. In order to assess the optimum conditions for differentiation of red and white muscle fibres a range of pre-incubation times (0 to 10 min) and pH values (pH 4·3 to 4·6 for acid-stable ATPase and pH 10·1 to 10·4 for alkali-stable ATPase) were used. For comparison, similar histochemical tests were carried out on frozen muscle sections of 11-month-old parr.

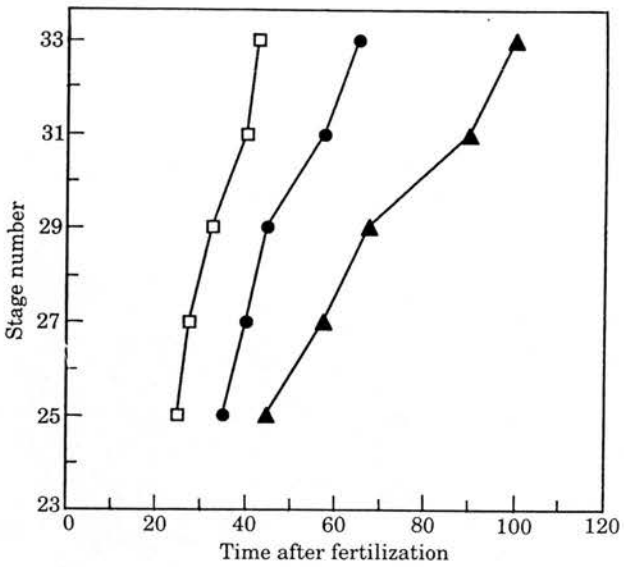

FIG. 2. Gorodilov stage numbers against days after fertilization for embryos at 5° C (▲), 8° C (●) and 11° C (□). Stage 33=hatching.

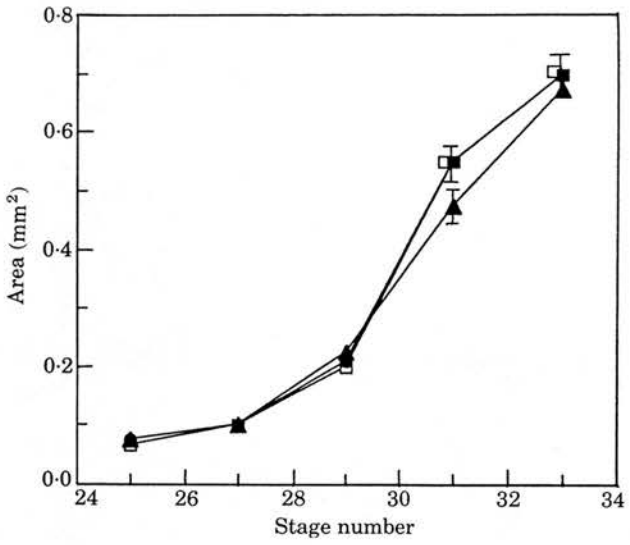

FIG. 3. Total white muscle cross-sectional area against stage number for embryos incubated at 5° C (▲), 8° C (●) and 11° C (□).

III. RESULTS

The time to hatching from fertilization was inversely proportional to incubation temperature (Fig. 2) being shortest (44 days) at 11° C, intermediate (64 days) at 8° C and longest (106 days) at 5° C. Both red and white muscle cross-sectional area (Fig. 3) increased with time after fertilization but at a given stage number, cross-sectional area was not different between temperature

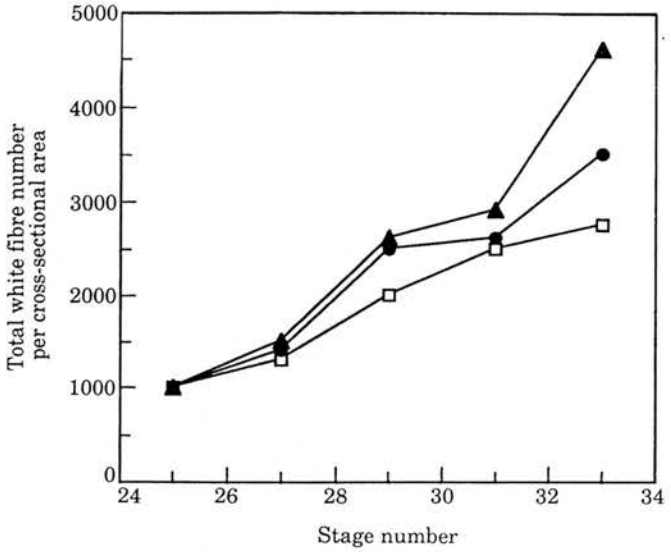

FIG. 4. Total white fibre number per cross-sectional area against stage number for embryos incubated at 5° C (▲), 8° C (●) and 11° C (□).

treatments. Furthermore, from stage 27 (when red fibres could be readily distinguished) the ratio of white to red muscle cross-sectional area was, both between stages and between temperature treatments, constant at approximately 20 : 1.

MUSCLE FIBRE NUMBERS AND SIZES

White muscle

Total white muscle fibre numbers per cross-sectional area (Fig. 4) were not significantly different between temperature treatments at stage 25. However, while fibre number increased with development at all three temperatures, the rate of fibre number increase (hyperplasia) was fastest at the lowest temperature and slowest at the highest temperature such that, at hatching, the total number of fibres per cross-section in embryos incubated at 5° C was almost double the number in embryos at 11° C; fibre number in 8° C embryos was intermediate. Conversely, at stage 33 mean myofibre area and mean myofibrillar area were greatest in embryos incubated at 11° C (Fig. 5), the percentage of difference in myofibrillar areas being greater than in myofibre areas.

Red muscle

Red muscle fibre number proved difficult to assess at stage 25 although there was an indication of a group of cells destined to develop into red muscle. At stage 27, there was no difference in red fibre number per cross-section between the three temperature groups [Fig. 6(a)]. However, by stage 33 the number of red fibres was significantly less in the 11° C embryos than the 8° C and less in 8° C than in 5° C embryos [Fig. 6(b)].

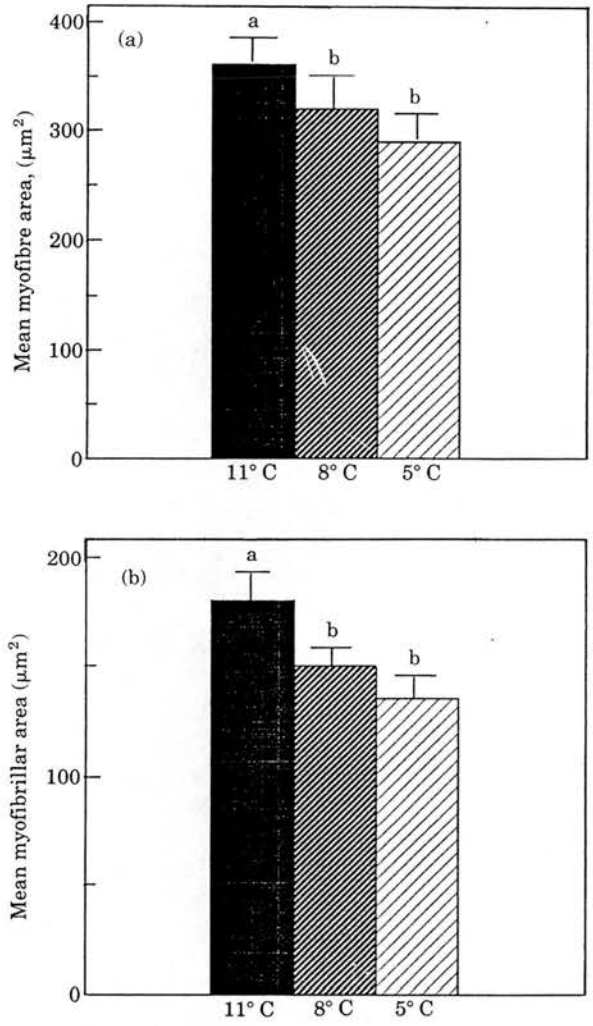

FIG. 5. (a) Mean white myofibre cross-sectional area and (b) mean myofibrillar cross-sectional area (in white myofibres) at stage 33 for each temperature. Error bars represent standard errors of the means. (Columns with different letters are significantly different at $P<0{\cdot}05$).

NUMBER OF NUCLEI

Total number of nuclei per cross-sectional area of white muscle did not differ between temperature treatment at stage 25 [Fig. 7(a)]. However, by stage 33 nuclear numbers were significantly higher in the 5° C embryos than in the 8 and 11° C embryos [Fig. 7(b)]. It was possible that the greater nuclear numbers in 5° C embryos might be directly related to the increased fibre number at this temperature. In order to assess this possibility the ratio of nuclei to fibre numbers was estimated. Figure 8 shows that at stage 33 this ratio was higher in the 11° C embryos.

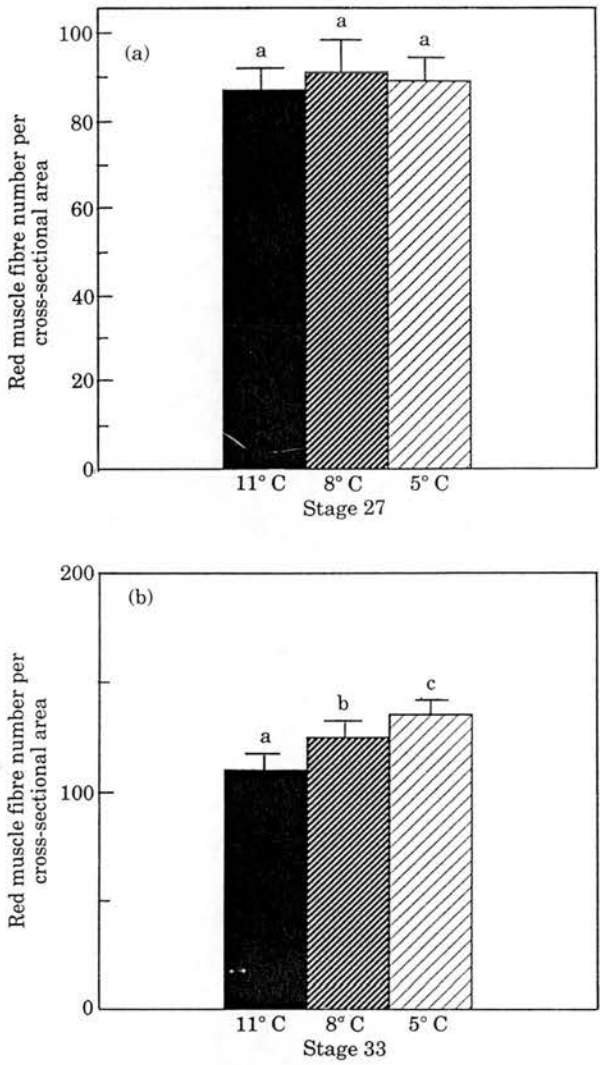

FIG. 6. Total red muscle fibre number at (a) stage 27 and (b) stage 33 for each temperature. Error bars represent standard errors of the means. (Columns with different letters are significantly different at $P<0{\cdot}05$).

MUSCLE HISTOCHEMISTRY

The optimum pre-incubation conditions for differentiating red and white muscle were shown to be pH 4·5 for 5 min (acid-stable ATPase) and pH 10·1 for 10 min (alkali-stable ATPase) (Table I). The optimum alkali pre-incubation inactivated red myofibres in all embryonic stages and in parr whereas white muscle exhibited intense staining. Interestingly, sub-optimal pH conditions (10·2, 10·3) produced a low intensity white fibre stain which showed a noticeable gradation in intensity from medial (0) to lateral (1) in many sections. The optimum acid pre-incubation inactivated white muscle in

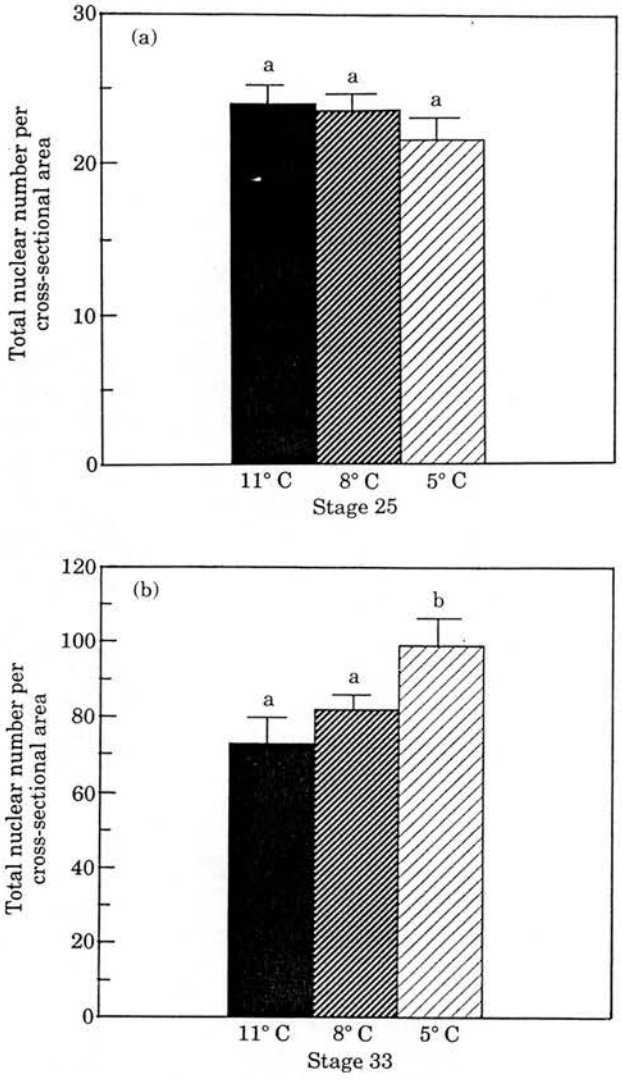

FIG. 7. Total nuclear number per cross-section at (a) stage 25 and (b) stage 33 for each temperature. Error bars represent standard errors of the means. (Columns with different letters are significantly different at $P<0{\cdot}05$).

parr and produced intense staining of red fibres. However, the same conditions in the embryos did not clearly differentiate the muscle fibre types.

Succinic dehydrogenase activity was low in embryonic fish but in the parr there was high activity in the red muscle with evidence of some superficial white fibres having a higher activity than the bulk.

No obvious differences could be detected between either embryonic stages or between temperature treatments for any enzyme activities (except for those already mentioned).

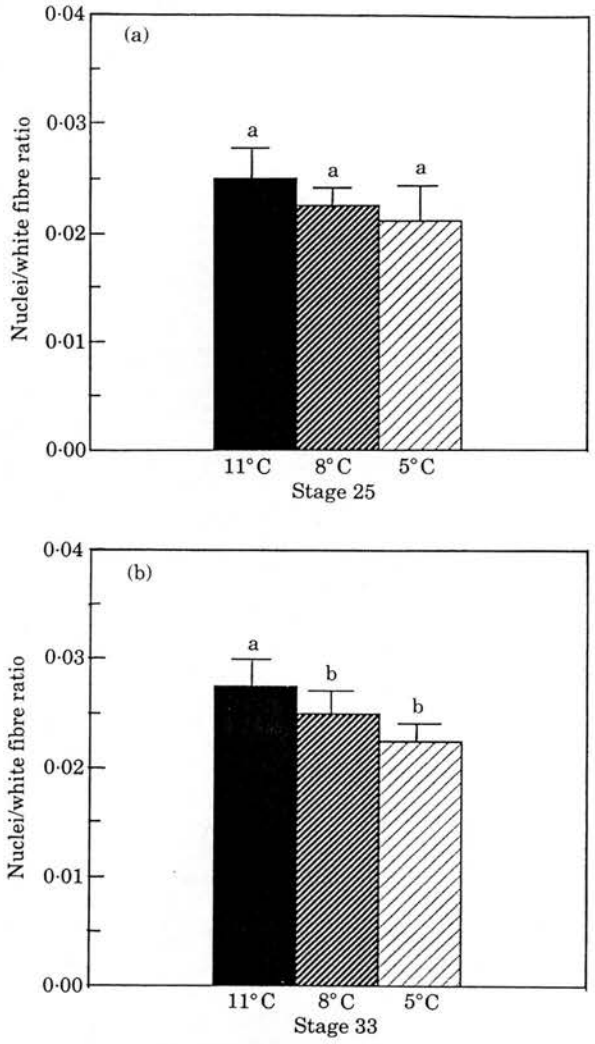

FIG. 8. Nuclei: White fibre ratio at (a) stage 25 and (b) stage 33 for each temperature. Error bars represent standard errors of the means. (Columns with different letters are significantly different at $P<0{\cdot}05$).

IV. DISCUSSION

Increased rearing temperatures rapidly increased the rate of development of the salmon embryos as shown by Hayes *et al.* (1953) and Marr (1966). At the time of hatching, which occurred earlier in the warmer embryos, total muscle cross-sectional area was similar for each temperature group, but the cellularity of the muscle was markedly different. The size of constituent muscle fibres was larger in warmer embryos and the effect was directly related to the temperature of development. The number of constituent fibres was lower in warmer embryos and, again, the extent of this effect was related to temperature. This confirms the earlier study of Stickland *et al.* (1988) which investigated the effect of two temperatures on cellularity of the white muscle. The present study has extended this to the red muscle in which a similar situation was found to occur.

TABLE I. Myosin ATPase histochemistry

	Pre-incubation time (min)	pH							
		4·3	4·4	4·5	4·6	10·1	10·2	10·3	10·4
Embryos	0	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
	3	0/0	0/0	0/0	0/0	0/3	0/1	0/1	0/0
	5	0/0	0/0	1/1	1/1	1/4	0/1	0/1	0/0
	10	0/0	0/0	1/1	1/1	0/4	1/1	0/0	0/0
Parr	5	1/1	2/1	3/1	2/1				
	10					0/4	1/1	1/1	1/1

Effect of time and pH of pre-incubation on staining intensity which was given a value of 0 (no stain) to 4 (very dark) for each section. The first number given refers to the staining intensity of red fibres; the second number to white fibres.

This finding is in contrast to the findings in postlarval fish that faster growth is associated with increased fibre hyperplasia (Weatherley & Gill, 1987; Higgins & Thorpe, 1990). The present results also differ from the situation in herring larvae where warmer temperatures were found to increase fibre hyperplasia relatively (Vieira & Johnston, 1992). One possible explanation may be that, in the salmon embryos, there is survival value in reaching the largest possible size and developmental stage before the yolk sac is depleted. Check & Hill (1970) concluded that reduced energy intake compromised nuclear division but not necessarily cell hypertrophy. Protein synthesis (or fibre hypertrophy) may therefore be a more energy-efficient method of increasing body size than nuclear division (which is one pre-requisite of fibre hyperplasia). Rapid post-larval growth occurs when nutritional level is not a limiting factor and the fish can fuel further growth by the provision of new fibres. It is possible that, in the herring larvae, availability of nutrients may not be a limiting factor as herring will feed and are free-swimming immediately after hatching (before the yolk sac is depleted). This is in contrast to salmon larvae which spend a post-hatch period buried in gravel and not feeding. The salmon larvae are almost totally inactive and so the role of muscle in protein storage may be more important than that of propulsion. Furthermore, the herring embryos of Vieira & Johnston's (1992) work were from spring spawning which would contain more yolk than from autumn spawnings (Blaxter, 1969). In this respect it would be of interest to study autumn spawning embryos in which it is more likely that the muscle cellularity may develop as in salmon embryos in response to different temperatures.

As already mentioned, protein synthesis appeared to be increased to a relatively greater extent than nuclear division at higher temperatures. This is emphasized by the greater differences in myofibrillar area (representing muscle protein) than in muscle fibre area. Nuclear numbers were lower in the warmer embryos at hatching. However, lower nuclear numbers were not the sole reason for reduced fibre hyperplasia in the warmer embryos as the number of nuclei to fibre ratio was greater in the warmer embryos. Clearly, however, increased nuclei were needed in the larger fibres.

It has often been recorded that cold acclimation in post-larval fish increases the proportion of red muscle (Johnston & Lucking, 1978). In the embryonic

salmon, temperature appeared to have no effect on the proportion of red to white muscle. However, the histochemical studies showed that the aerobic enzyme succinic dehydrogenase was not significantly active in the salmon embryos. This implies that the metabolic profiles of the musculature had not differentiated and so it is not surprising that no effect was seen at this stage.

The difference between embryonic and older fish in staining intensity for the acid and alkali-stable myosin ATPase points to the possible existence of different myosin isoforms in embryonic and mature myofibres. This situation has been demonstrated in Arctic charr (*Salvelinus alpinus* L.) by Martinez *et al.* (1991). The difference in staining intensity across the muscle from medial to lateral aspect may also be a feature of the most lateral fibres being the newest to form as shown by Stickland *et al.* (1988) in salmon embryos and by Waterman (1969) in *Brachydanio rerio* Hamilton. The newer fibres may exhibit different myosin isoforms to older fibres even in the same section.

Thanks are due to Mr M. S. Miles and his staff at the Almondbank Smolt Station for careful husbandry of the salmon, to P. Sands for assistance with much of the histochemistry, and to Mr Nathanailides for helpful discussions.

References

Blaxter, J. H. S. (1969). Development: eggs and larvae. In *Fish Physiology*, Vol. III (Hoar, W. S. & Randall, D. J., eds), pp. 178–241. New York: Academic Press.

Cheek, D. B. & Hill, D. E. (1970). Muscle and liver cell growth: role of hormones and nutritional factors. *Federation Proceedings* **29,** 1503–1509.

Gorodilov, Yu. N. (1983). Stadii embryonalnogo razvitiya atlanticheskogo lososya *Salmo salar* L. II. Opisaniye i khronologiya. *Sbornik Nauchnykh Trudov GosNIORKh* **200**, 107–126. Stages of embryonic development in Atlantic salmon, *Salmo salar* L. II. Description and chronology. Translation: Thorpe, J. E. *Scottish Fisheries Research Translation* **19,** 1–34.

Guth, L. & Samaha, F. J. (1970). Procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28,** 365–367.

Hayes, F.R., Pelluet, D. & Gorham, E. (1953). Some effects of temperature on the embryonic development of the salmon (*Salmo salar*). *Canadian Journal of Zoology* **31,** 42–51.

Higgins, P. J. (1990). The histochemistry of muscle in juvenile Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology* **37,** 521–529.

Higgins, P. J. & Thorpe, J. E. (1990). Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology* **37,** 505–519.

Johnston, I. A. & Lucking, M. (1978). Temperature induced variation in the distribution of different types of muscle fibre in the goldfish (*Carassius auratus*). *Journal of Comparative Physiology* **124,** 111–116.

Johnston, I. A., Patterson, S., Ward, P. S. & Goldspink, G. (1974). The histochemical demonstration of myofibrillar adenosine triphosphatase activity in fish muscle. *Canadian Journal of Zoology* **52,** 871–877.

Jones, B. (1979). A review of three resin processing techniques applicable to light microscopy. *Medical Laboratory Sciences* **36,** 249–267.

Krogh, A. (1914). On the influence of the temperature on the rate of embryonic development. *Allgemeine Physiologie* **16,** 163–177.

Marr, D. H. A. (1966). Influence of temperature on the efficiency of growth of salmonid embryos. *Nature* **212,** 957–959.

Martinez, I., Christiansen, J. S., Ofstad, R. & Olsen, R. L. (1991). Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Arctic charr *Salvelinus*

alpinus (L). Sequential expression of different myosin heavy chains during development of the fast white skeletal muscle. *European Journal of Biochemistry* **195,** 743–753.

Nachlas, M. M., Tsou, K. C., de Sonza, E., Cheng, S. S. & Seligman, A. M. (1957). Cytochemical demonstration of succinic dehydrogenase by the use of new p-nitrophenyl substituted ditetrazale. *Journal of Histochemistry and Cytochemistry* **5,** 420–436.

Ontell, M. (1974). Muscle satellite cells: a validated technique for light microscope identification and a quantitative study of changes in their population following denervation. *Anatomical Record* **178,** 211–228.

Stickland, N. C., White, R. N., Mescall, P. E., Crook, A. R. & Thorpe, J. E. (1988). The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L.). *Anatomy and Embryology* **178,** 253–257.

Vieira, V. C. A. & Johnston, I. A. (1992). Influence of temperature on muscle-fibre development in larvae of the herring *Clupea harengus. Marine Biology* **112,** 333–341.

Waterman, R. E. (1969). Development of the lateral musculature in the teleost, *Brachydanio rerio*: a fine structural study. *American Journal of Anatomy* **125,** 457–492.

Weatherley, A. H. & Gill, H. S. (1987). *The Biology of Fish Growth.* London: Academic Press.

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The growth of Atlantic salmon (*Salmo salar* L.) myosatellite cells in culture at two different temperatures

T. W. Matschak and N. C. Stickland

The Royal Veterinary College, Royal College Street, London NW1 0TU (England, UK)
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Abstract. Temperature is known to affect fish growth, and in Atlantic salmon there is an influence on muscle cellularity. Primary muscle cell culture makes it possible to investigate direct effects of temperature on myogenic cells. Salmon myosatellite cells were cultured for the first time in this study. The cells were cultured at either 5 °C or 11 °C. Increased temperature led to an increase in differentiation rate and especially hypertrophic growth ($Q_{10} = 4.0$). No nuclear proliferation was evident in the satellite cell population isolated at either temperature. This may be due to the presence of different subpopulations of myogenic cells at different developmental ages or the presence of indirect factors in vivo.
Key words. Salmon; satellite cells; cell culture; temperature.

Fish muscle constitutes 30–80% of the body mass of the animal[1] and growth is closely linked to muscle growth[2]. In mammalian muscle, growth by hyperplasia, that is recruitment of new muscle fibres, ceases to play a role at around the time of birth[3,4]. In fish, however, both hyperplastic growth and muscle fibre hypertrophy, the increase in size of individual fibres, are important throughout almost the entire life of the animal[5–8].

The growth rate of salmonids is influenced by environmental temperature. Mathers et al.[9] found that fed trout at 10 °C and at 15 °C grow more rapidly than at 5 °C, and this is concomitant with a higher protein retention rate. This is consistent with an increase in protein synthesis rates after acclimatisation to a higher temperature[9,10].

There is evidence from work on pre-hatch salmon that temperature does not affect all growth processes equally. In Atlantic salmon, it has been found that embryos not only develop faster and exhibit a higher growth rate at higher temperatures, but that the muscle cellularity is distinctly influenced by incubation temperature. An increase in temperature results in the fish having fewer but larger muscle fibres by the time of hatching[11,12]. This constitutes a relative increase in growth by hypertrophy as opposed to hyperplasia when compared with fish grown at a lower temperature. In contrast, faster growth in post-hatch fish has been found to be associated with an increase in fibre number[1,8,13,14]. In juvenile salmon also, faster growth is associated with an increase in fibre number[15].

The satellite cell is generally regarded as the source of muscle nuclei for fibre hypertrophy and the addition of new fibres in growth[16,17]. In recent years, cell culture techniques have become available for fish satellite cells[18,19]. The difference in growth mechanism before and after hatching may be due to indirect factors. However, given the potential significance of this cell type in fish muscle growth it is of importance to assess the presence of satellite cells in salmon and to investigate the direct effect of temperature, one of the most important environmental factors in muscle growth of poikilotherms, on these cells. In this study, cell culture was applied to Atlantic salmon satellite cells and the direct effect of temperature on cell volume and nuclear numbers determined.

Materials and methods

Animals. Fish were reared at the DAFS Freshwater Fisheries Laboratory, Pitlochry, Scotland. Juveniles of an average length of 7.2 cm were sent to London and the cells isolated there.

Isolation of myosatellite cells. The method of Koumans et al.[19] was modified. The fish were stunned by a blow to the head and killed by decapitation. In order to sterilise the surface of the fish, they were immersed in 70% ethanol for 30 s, the mucus was scraped off and the fish were sprayed with 70% ethanol. Axial white muscle was excised under sterile conditions and collected in 90% Dulbecco's modified Eagle medium containing non-essential amino acids (DMEM; Gibco BRL, Paisley, United Kingdom) supplemented with 15% Horse serum (HS; Gibco) and PenStrep (50 U/ml penicillin, 100 µg/ml streptomycin; Sigma, Poole, United Kingdom). The medium was buffered using HEPES (Sigma) at a concentration of 10 mM. The tissue was then thoroughly minced, spun down (300 g, 5 min, 4 °C) and washed twice in medium without serum supplement.

The fragmented tissue was then subjected to a digestion in 90% DMEM containing 0.2% collagenase (Sigma)

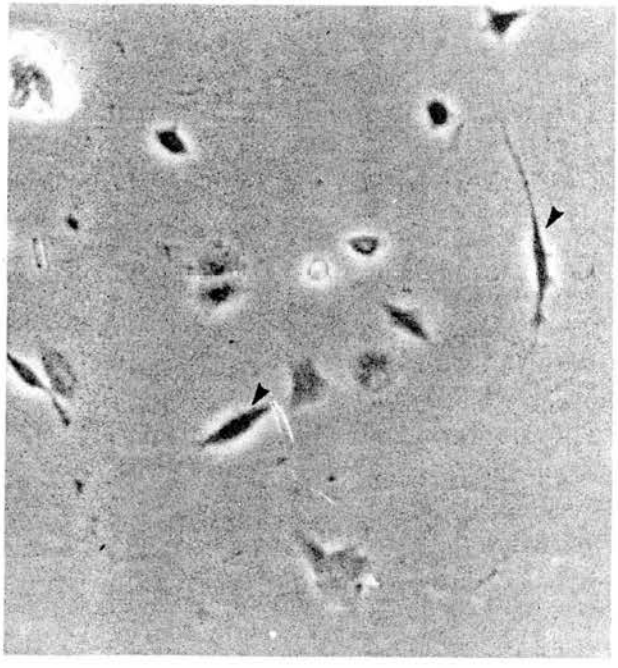


Figure 1. Atlantic salmon satellite cell culture after 24 h at 11 °C. Cells showing the typical spindle shaped morphology of a satellite cell are shown (arrowhead). Phase contrast microscopy; magnification = 200 ×.

for 90 min at 11 °C under gentle agitation (5 ml of solution per g tissue excised). The resulting suspension was washed once in 90% DMEM, triturated five times through a 10 ml pipette and washed again (300 g, 5 min, 4 °C).

The resulting pellet was resuspended in a 0.1% trypsin (Sigma, Poole, United Kingdom) solution (5 ml per g of tissue excised) and incubated for 30 min at 11 °C with gentle agitation. The suspension was centrifuged for 1 min at 300 g, the supernatant aspirated and collected in two volumes of ice-cold medium containing serum (complete medium). The pellet was subjected to another trypsin digestion step (2.5 ml of solution per g of excised tissue) and finally diluted in 2 volumes of complete medium.

All the resulting cell suspensions were spun down (300 g, 15 min, 4 °C). The pellet was resuspended in ice-cold complete medium, triturated and filtered through firstly 40 µm and then 20 µm Nytex filters. The cells were then washed once more and plated at a concentration of 2×10^6 cells/ml.

The myosatellite cells were purified by adhesion to laminin coated culture flasks as described by Koumans et al.[11]. The combined cells from 10 fish were left to adhere for 20 min at 11 °C without disturbance. 8×10^6 cells were plated per 25 cm² culture flask. The supernatant containing non-adherent cells was then aspirated and complete medium added.

Cell culture. After isolation and purification, the cells were incubated at either 5 °C or 11 °C in cooled incubators (LMS, Sevenoaks, United Kingdom). The culture medium (90% DMEM supplemented with 15% HS) was changed every ten days and no excessive vacuolisation indicating undernourishment was observed. The growth of the cells was monitored visually every two days.

Analysis. Pilot studies had shown that the cells develop and differentiate much faster at the higher temperature. The cultures incubated at 11 °C were fixed in absolute methanol for 5 min and stained with Leishman's stain (Merck, Lutterworth, United Kingdom) after 20 days of culture when most cells appeared to have fused as judged by visual inspection. The cultures incubated at 5 °C were fixed and stained in the same way after 48 days. After this period of time the 5 °C cultures showed the same appearance in visual microscopic inspection as the 11 °C cultures at 20 days. Briefly, the substratum was covered with the stain, after 1 min an equal amount of phosphate buffer (pH 6.8; Gurr; Merck, Lutterworth, United Kingdom) was added and the cells were incubated for 15 min at room temperature. The stain was then washed off with more phosphate buffer until the cells appeared just pink. Phosphate buffered saline (PBS) containing 0.1% sodium azide was added and the cells were photographed.

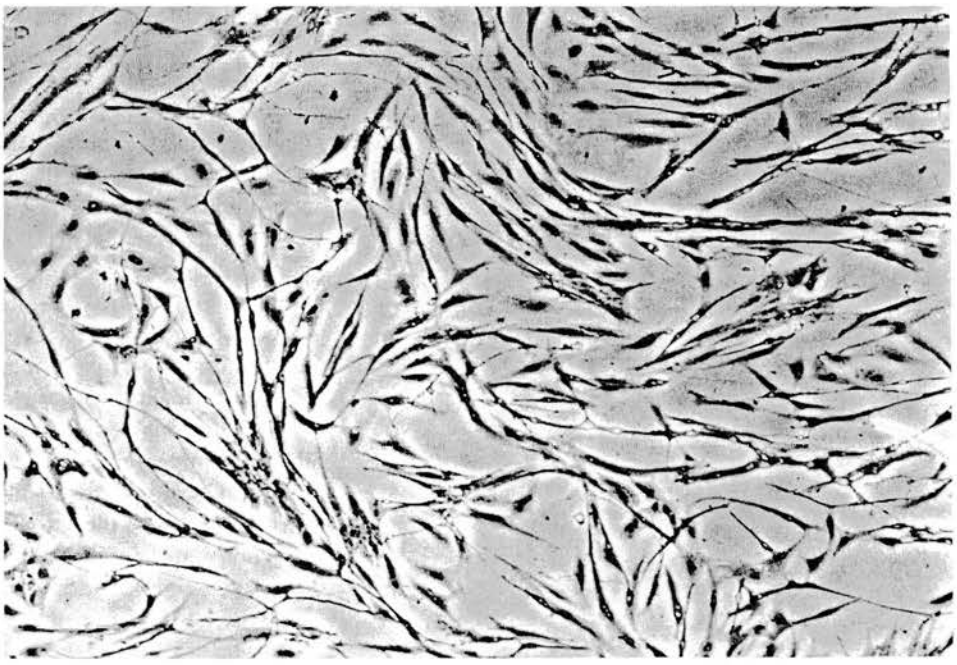
Ten randomly chosen fields of vision from the fixed and stained cultures, each corresponding to an area of 0.13 mm², for each temperature were used to determine nuclear numbers and cell size with a Seescan image analyser. For a comparison of the two temperature treatments at 20 days unstained cultures were used. The original number of adherent cells was determined also from unstained cultures.

Antibody staining. To verify that the cultured cells produced muscle specific protein, the cells were stained using an anti-myosin heavy chain mouse monoclonal antibody (83b6; a kind gift from Dr. G. K. Dhoot). The cells were fixed for 5 min in −20 °C cold 50% acetone/50% ethanol. Incubation took place in PBS containing 0.5% bovine serum albumin at a dilution of 1:200. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako Ltd. High Wycombe, United Kingdom) was used as the secondary antibody. Chromogen, H_2O_2, and substrate mixture (AEC) from a DakoPAP kit (Dako Ltd.) were applied to visualise the bound antibody.

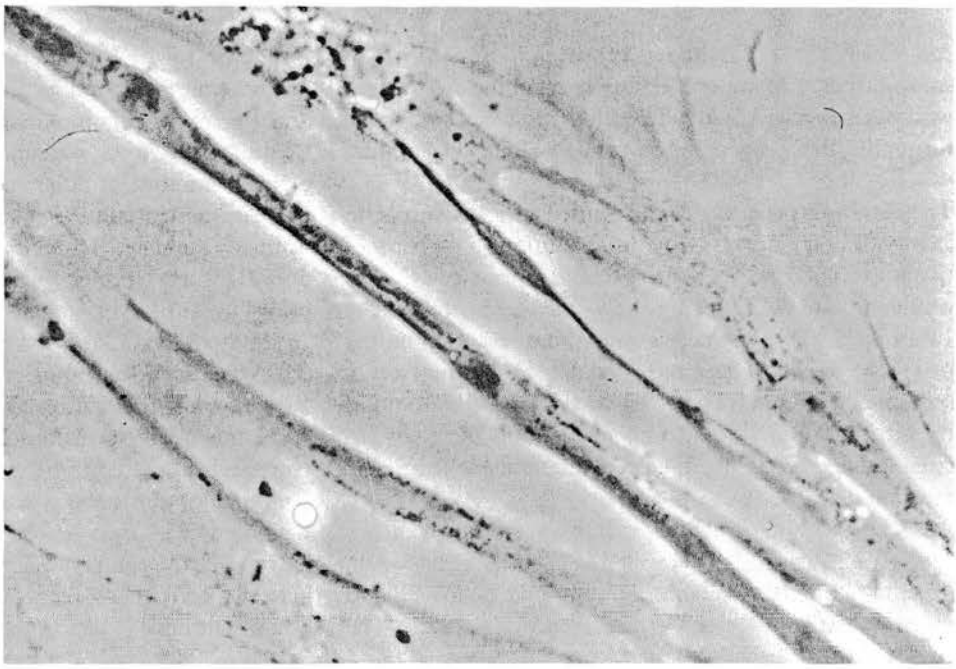
Statistics. Nuclear numbers and cell sizes were compared using a Student *t*-test.

Results

Morphology and immunocytochemistry. Cells exhibiting the spindle shaped morphology of satellite cells[18,19] were first detected after 24 h of culture (fig. 1). Fusion of cells was first observed after 5 days of culture for 11 °C and 9 days of culture at 5 °C. At least 50% of the cells had fused after 13 days at the higher temperature and after



A

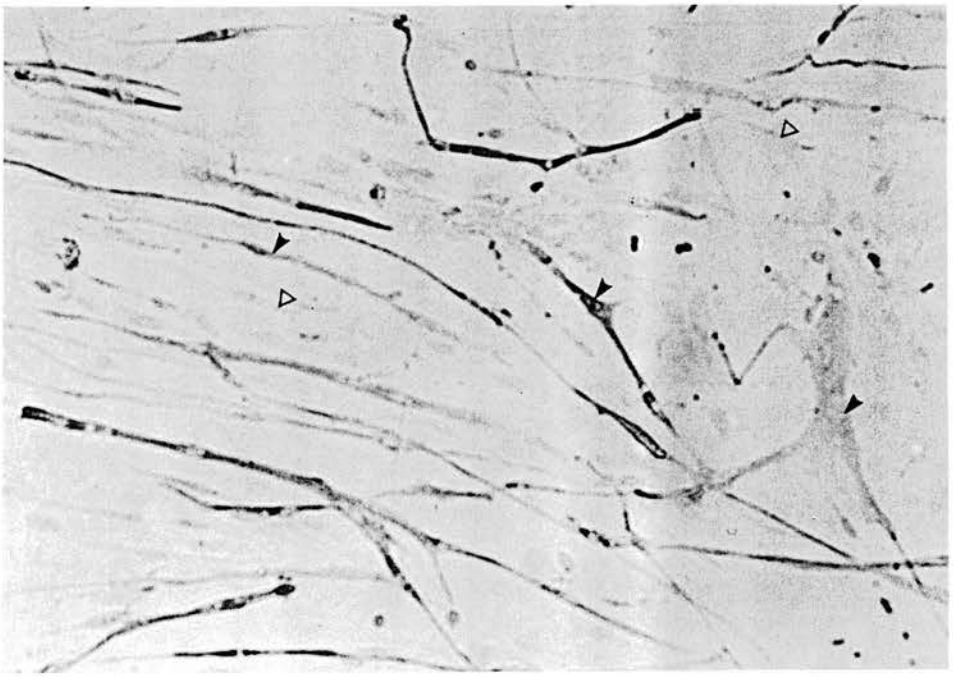


B

Figure 2. Atlantic salmon satellite cell culture.
A) Appearance of cultures incubated at 5 °C for 48 days after isolation. Almost all of the cells had undergone fusion. Phase contrast microscopy; magnification = 80 ×.
B) Myotubes formed from the isolated satellite cells cultured for 20 days at 11 °C. Note the myotube containing several nuclei. Phase contrast microscopy; magnification = 400 ×.

22 days at 5 °C with virtually all cells having fused after 20 days and 48 days, at 11 °C and 5 °C respectively (fig. 2A). The formed myotubes showed the typical morphology described in the literature (fig. 2B).

In addition to the morphological criteria, the ability of the cells to synthesise muscle protein, in this case myosin heavy chain, was confirmed by immunocytochemistry. The reactivity of the antibody was tested on frozen sections of juvenile salmon. 83b6 was found to bind to red and white muscle fibres but not to any other tissue in the fish (results not shown). In the cultures the antibody did bind to the majority but not to all myotubes (fig. 3A). Cross-striated cells could be detected after the antibody staining (fig. 3B).



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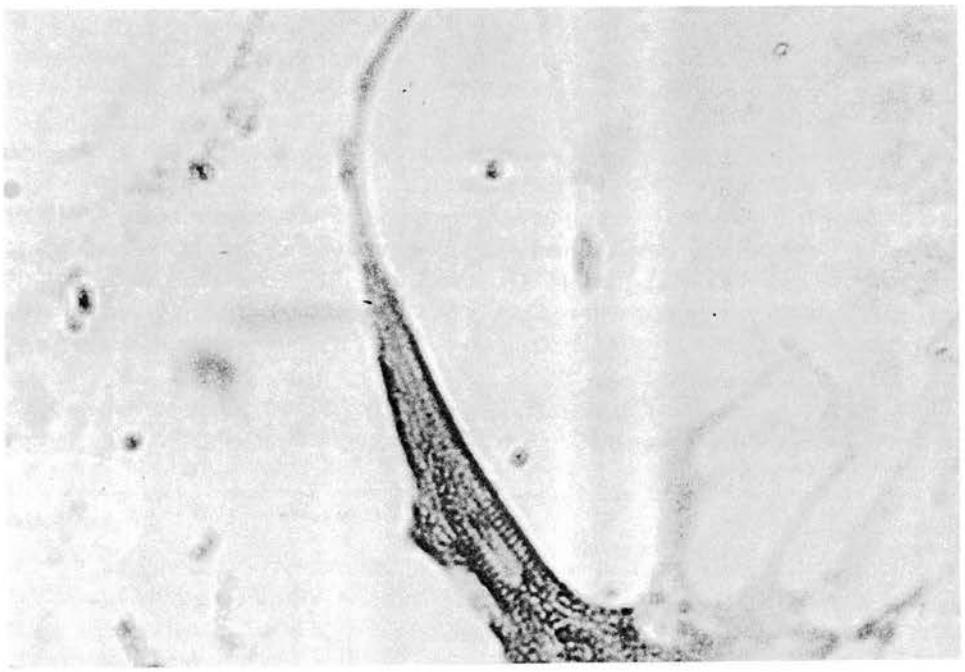


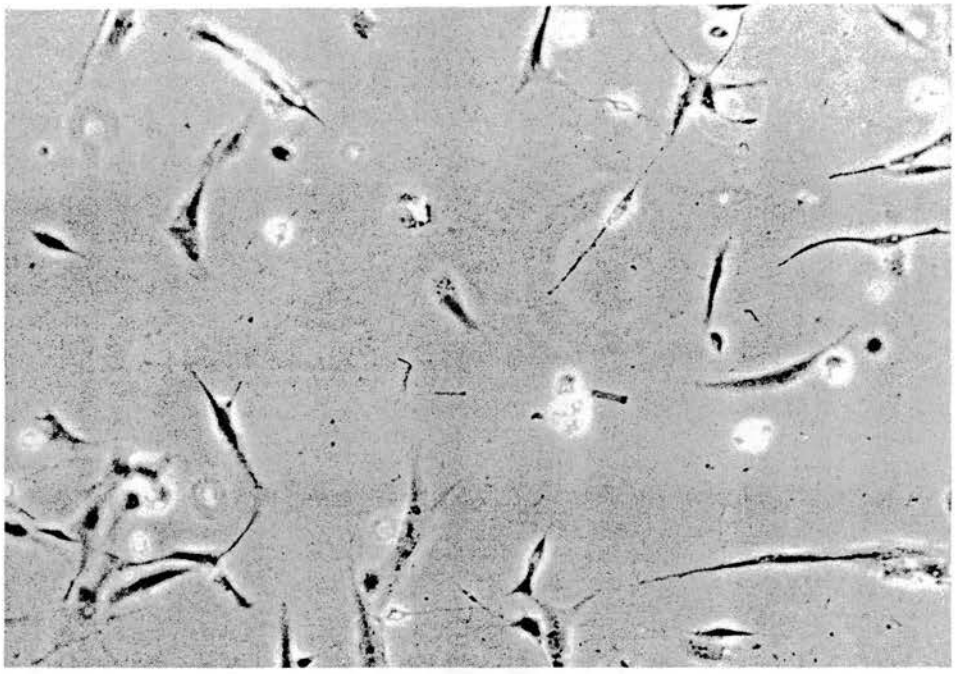
B

Figure 3. Binding of mouse monoclonal antibody specific for myosin heavy chain (83b6) to cultured Atlantic salmon satellite cells.
A) Cells cultured at 5 °C for 50 days. Bound antibody is shown as a dark stain. Some myotubes with bound antibody are emphasised with filled arrowheads; some antibody-negative cells are highlighted with open arrowheads. Magnification = 200 ×.
B) Strongly stained myotube from the above culture. Note the cross-striations visible within the cell. Magnification = 600 ×.

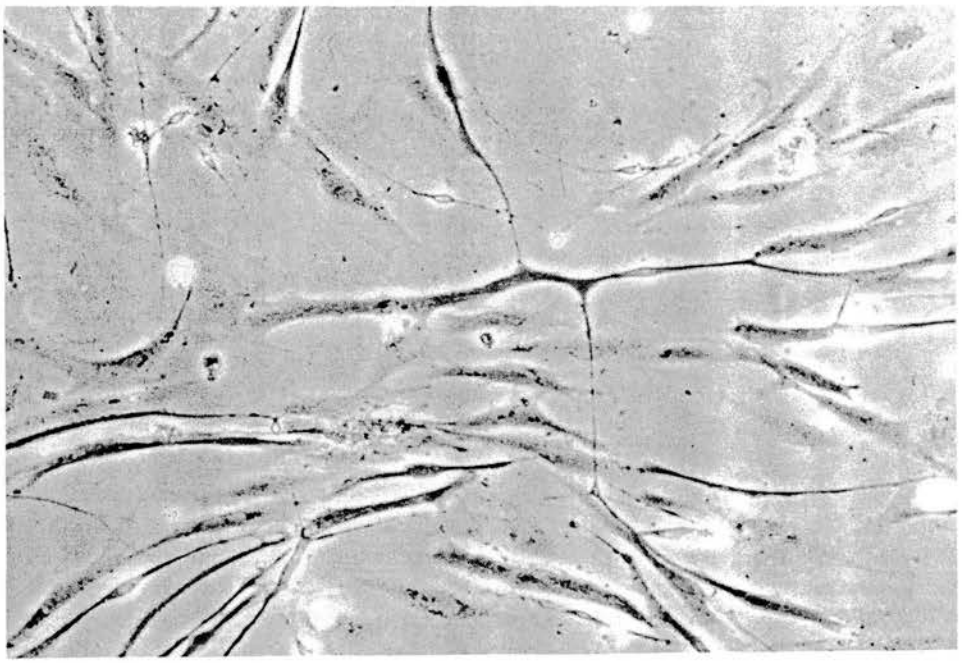
Nuclear numbers and cell size. The results are summarised in the table. The number of cells that attached to the laminin coated substratum after 20 min at 11 °C was 2.6×10^5 cells per g of tissue. This represents about 5% of the total number of cells (30×10^6) plated. After 20 days of culture there was no statistically significant difference in nuclear numbers between the two temperature treatments ($p = 0.855$). However, the development and growth in cell size had proceeded at a much faster speed at the higher temperature. This is reflected in a highly significant difference in the area occupied by the cells ($p < 0.001$) and is also clearly visible (fig. 4A, C). The cultures incubated at 5 °C were grown on to 48 days, by which time the appearance of the culture was

Table. Percentage of substratum occupied by cells and nuclear numbers per mm². Standard errors are given. nd = not determined.

Temp., Age	0 d	5 °C, 20 d	11 °C, 20 d	5 °C, 48 d	11 °C, 20 d
Area (%)	0.3 ± 0.03	6.4 ± 0.8	29.4 ± 0.8	40.2 ± 4.8	38.6 ± 4.1
p		< 0.001		0.797	
Number/mm²	156 ± 6	118 ± 6	117 ± 5	165 ± 10	167 ± 9
p		0.855		0.857	

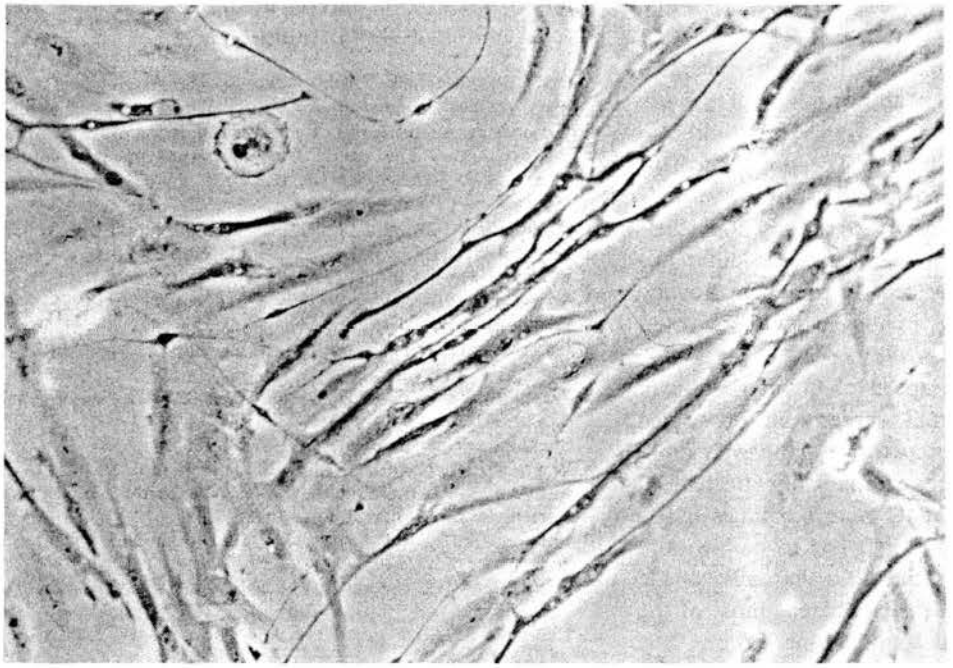


A



B

Figure 4. Parts A, B.
(for legend see part C on following page).



C

Figure 4. Satellite cell culture from Atlantic salmon Phase contrast microscopy was used.
A) Cells cultured at 5 °C for 20 days.
B) Cells cultured at 5 °C for 48 days.
C) Cells cultured at 11 °C for 20 days.
Note that *B*) and *C*) are virtually indistinguishable in appearance and that virtually all cells have fused in these cultures. Magnification = 200 ×.

identical to the one incubated at the higher temperature for 20 days (fig. 4C). Image analysis revealed that there was still no statistically significant difference in nuclear numbers between the two temperatures at this stage ($p = 0.857$). The nuclear numbers determined in this case are higher than above. This is due to the fact that unstained cultures were used for comparing the cultures at day 20 whereas stained cells were analyzed when day 20 at 11 °C was compared with day 48 at 5 °C. This led to an underestimate of the actual number present in the former case. Interestingly, the cell sizes at 5 °C had increased to an extent that there was no significant difference between the two temperature treatments any more ($p = 0.797$; 5 °C at 48 days and 11 °C at 20 days).

Discussion

Salmon satellite cells were cultured for the first time in this study. Myosatellite cells are small, spindle-shaped cells found under the basal lamina of muscle fibres and are generally held responsible for the formation of new muscle fibres as well as for supplying nuclei to existing fibres undergoing growth (mammals[16]; fish[17]). Their presence has been confirmed in many fish species (e.g. trout[18]; eel[20]; carp[21]).

Satellite cells have been previously cultured from carp[13,19] and from trout[18]. In this study, the cultured cells were identified as satellite cells on the basis of the following criteria: 1) The morphology of the cells was in accordance with that found for carp and for trout. 2) The vast majority of the cells isolated proceeded to fuse and formed myotubes. 3) Myofibrillar protein was demonstrated in a substantial number of these myotubes formed (see fig. 3A). This further demonstrates the involvement of myosatellite cells in the development of fish muscle. Interestingly, a yield of cells similar to that found in the carp[13] was achieved in the salmon.

This is the first study in which an attempt was made to analyse the direct influence of temperature on myosatellite cells. The analysis concentrated on two factors, namely nuclear proliferation and increase in cell size. Temperature influences the growth rate of fish muscle in salmonids[9], and muscle growth is associated with an increase in nuclear numbers[22,23]. However, in this study it was found that no proliferation took place in the myosatellite cell population isolated. This is consistent with the findings of Koumans et al.[19] who found only a very small proportion of proliferating cells in the carp, as measured by BrdU incorporation 17 h after isolation, and no change in the number of muscle nuclei. Powell et al.[18] found no obvious proliferation in the trout.

In contrast, it has been found that in older carp a higher percentage of myosatellite cells show proliferation[24]. Koumans et al.[24] speculate that this is due to two subpopulations of satellite cells being present in fish. One subpopulation, which exhibits a postmitotic nature, would be responsible for hyperplasia and is therefore found at stages growing mainly by this mechanism. The other subpopulation would be involved in growth by muscle fibre hypertrophy. In salmon growth occurs mainly by hyperplasia in fish longer than 6.5 cm[15] and the satellite cells isolated in this study may therefore

represent the above mentioned postmitotic subpopulation.

However, an alternative explanation may be the presence of indirect factors, such as hormones or growth factors, leading to proliferation in vivo. This could also explain the findings in the carp[19]. The presence of at least some proliferation at isolation is shown by BrdU incorporation. These cells seem to rapidly lose their proliferative capacity, however, and undergo differentiation. With only 10% of the cells in the carp showing BrdU incorporation a change in nuclear numbers would thus not become apparent.

Temperature influenced the speed of differentiation in the cultures. Fusion occurred earlier and proceeded much faster at the higher temperature. However, more interestingly, cell size was very dramatically affected. At day 20 the cells raised at 11 °C were 4.6 times larger than the cells incubated at 5 °C. It has to be borne in mind, however, that after 20 days less than 50% of the lower temperature cells had fused whereas most of the higher temperature cells had undergone fusion. The large difference found may therefore represent a developmental difference, rather than a difference in the hypertrophic growth of myofibres. When cultures containing mostly differentiated myotubes are compared, i.e. after 20 days for 11 °C and after 48 days for 5 °C, a Q_{10} of 4.0 can be calculated. This is not dissimilar to the Q_{10} of 5.75 found for total protein growth in fed rainbow trout[9] and may suggest a direct temperature control of hypertrophic growth. The result clearly shows that a surprisingly high increase in hypertrophic growth of myotubes can be effected directly by temperature.

In summary this study has shown that, in the salmon, there is an absence of proliferative activity in at the least a subset of myosatellite cells. This may be due to the absence of indirect factors necessary for proliferation. Temperature had a very pronounced direct effect on fusion, differentiation and hypertrophic growth of satellite cells and myotubes in culture.

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1 Weatherly, A. H., and Gill, H. S., Experientia *41* (1985) 353.
2 Fauconneau, B., Aguirre, P., and Blanc, J. M., Comp. Biochem. Physiol. *97C* (1990) 345.
3 Rayne, J., and Crawford, G. N. C., J. Anat. *119* (1975) 347.
4 Stickland, N. C., J. Anat. *132* (1981) 557.
5 Greer-Walker, M., J. Cons. perm. int. Explor. Mer *33* (1970) 228.
6 Weatherly, A. H., Gill, H. S., and Rogers, S. C., Can. J. Zool. *57* (1979) 2385.
7 Stickland, N. C., J. Anat. *137* (1983) 323.
8 Weatherly, A. H., and Gill, H. S., J. Fish Biol. *25* (1984) 13.
9 Mathers, E. M., Houlihan, D. F., McCarthy, I. D., and Burren, L. J., J. Fish Biol. *43* (1993) 245.
10 Loughna, P. T., and Goldspink, G., J. expl Biol. *118* (1985) 267.
11 Stickland, N. C., White, R. N., Mescall, P. E., Crook, A. R., and Thorpe, J. E., Anat. Embryol. *178* (1988) 253.
12 Usher, M. L., Stickland, N. C., and Thorpe, J. E., J. Fish Biol. *44* (1994) 953.
13 Koumans, J. T. M., Akster, H. A., Booms, R. G. H., Lemmens, C. J. J., and Osse, J. W. M., Am. J. Anat. *192* (1991) 418.
14 Vieira, V. L. A., and Johnston, I. A., Mar. Biol. *112* (1992) 333.
15 Higgins, P. J., and Thorpe, J. E., J. Fish Biol. *37* (1990) 505.
16 Moss, P., and Leblond, C. P., Anat. Rec. *170* (1971) 421.
17 Bone, Q., in: Fish Physiology, vol. VII, p. 361. Eds W. S. Hoar and D. J. Randall. Academic Press, New York 1978.
18 Powell, R. L., Dodson, M. V., and Cloud, J. G., J. expl. Zool. *250* (1989) 333.
19 Koumans, J. T. M., Akster, H. A., Dulos, G. J., and Osse, J. W. M., Cell Tiss. Res. *261* (1990) 173.
20 Willemse, J. J., and van den Berg, P. G., J. Anat. *125* (1978) 447.
21 Akster, H. A., Neth. J. Zool. *33* (1983) 164.
22 Cardasis, C. E., and Cooper, G. W., J. expl Zool. *191* (1975) 347.
23 Koumans, J. T. M., Akster, H. A., Witkam, A., and Osse, J. W. M., J. Fish Biol. *43* (1993) 69.
24 Koumans, J. T. M., Akster, H. A., Booms, R. G. H., and Osse, J. W. M., Differentiation *53* (1993) 1.

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Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (*Salmo salar*)

C. Nathanailides, O. Lopez-Albors, and N.C. Stickland

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Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (*Salmo salar*)

C. Nathanailides, O. Lopez-Albors, and N.C. Stickland

Abstract: Previous work has shown that higher incubation temperatures produce newly hatched salmon (*Salmo salar*) with fewer but larger muscle fibres than salmon incubated at colder temperatures. Our purpose was to study the effect of differing incubation temperatures on the development of muscle cellularity in posthatch salmon. Eggs from a single pair of Atlantic salmon were incubated at either the stream ambient temperature (fluctuating around 5°C prehatch and gradually rising to around 10°C posthatch) or at 11°C. From each group, samples were taken at hatching, first feeding, and at 3 weeks after first feeding. During the period of exogenous feeding under study, the ambient group grew faster. The number of muscle fibres remained lower in the 11°C fish but there was more muscle fibre hypertrophy in the ambient group so that the difference in muscle fibre size seen at hatching was eliminated by 3 weeks after first feeding. It is suggested that reduced number of nuclei in the 11°C fish at hatching may contribute to the relatively reduced fibre hypertrophy in these fish. The results indicate that embryonic myogenesis can affect the posthatch growth of salmon at least up to 3 weeks after first feeding.

Résumé : Des travaux antérieurs ont démontré que les températures d'incubation plus élevées produisent des alevins de saumon atlantique (*Salmo salar*) pourvus de fibres musculaires moins nombreuses, mais plus grosses, comparativement aux saumons incubés à des températures plus basses. Nous avons examiné l'effet d'une variation des températures d'incubation sur le développement des cellules musculaires des saumons, après l'éclosion. Les oeufs d'une seule paire de saumons atlantiques ont été placés à incuber soit à la température ambiante du cours d'eau (oscillant aux alentours de 5°C avant l'éclosion et augmentant par la suite graduellement jusqu'à 10°C), soit à 11°C. Des échantillons ont été prélevés dans chaque groupe à l'éclosion, au début de l'alimentation, et 3 semaines plus tard. Pendant la période de nutrition exogène faisant l'objet de l'étude, le groupe placé à la température ambiante a montré une croissance plus rapide. Le nombre de fibres musculaires est demeuré inférieur chez les poissons élevés à 11°C, mais les fibres étaient plus grosses chez les sujets placés à la température ambiante, et la différence observée dans la taille des fibres musculaires au moment de l'éclosion avait disparu 3 semaines après le début de l'alimentation. Il est possible que le nombre réduit de noyaux, au moment de l'éclosion, chez les poissons placés à 11°C, ait contribué à la réduction relative de l'hypertrophie des fibres chez ces poissons. Ces résultats démontrent que la myogenèse peut influer sur la croissance des jeunes saumons au moins jusqu'à 3 semaines après le début de l'alimentation.

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roduction

asing temperature within the thermal range of a species speed up embryonic development. The development tlantic salmon (*Salmo salar*) embryos at 10°C can be e as fast as that of embryos incubated at 5°C (Usher et al. 1994). This fact can be used in salmon hatcheries to increase production and start the posthatch growing season earlier (Farmer et al. 1990). There is some evidence to suggest that an optimum temperature regime for the survival of fish embryos is one that is more closely related

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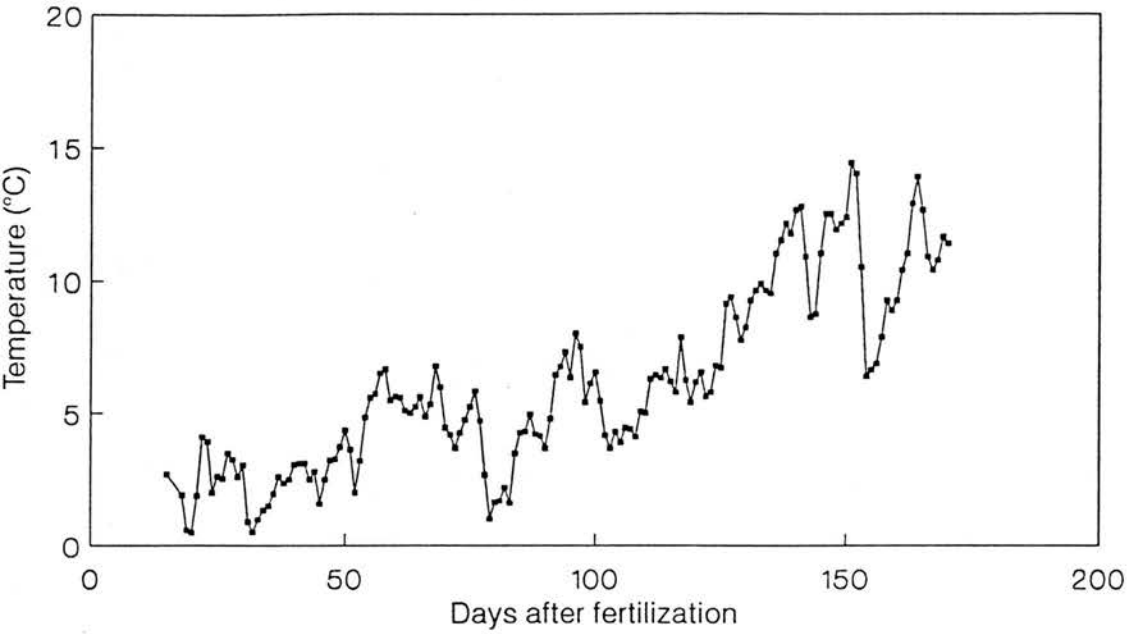
2401

. **Nathanailides[1] and N.C. Strickland.** Department of Veterinary Basic Sciences, The Royal Veterinary College, University London, Royal College Street, London, NW1 0TU, U.K.
. **Lopez-Albors.** Department of Anatomy and Embryology, University of Murcia Veterinary School, 30071 Murcia, Spain.
Author to whom all correspondence should be addressed.

Fig. 1. The ambient temperature of Almondbank salmon hatchery from the day of fertilization up to 3 weeks after first feeding for the ambient group.



to the natural environmental conditions of embryogenesis for each species (Hokanson and Kleiner 1974; Luczynski 1985). During embryonic development, temperature can affect the organogenesis and meristic features. Although the rate of development and growth is increased, the number of vertebrae (Klinkhart et al. 1987) and the total number of muscle fibres (Stickland et al. 1988) on hatching are decreased at high incubating temperature. By contrast in older fish, faster growth is associated with increased muscle fibre hyperplasia (Weatherley and Gill 1987). Such correlation is less evident in very young posthatch stages of salmonoid species (Weatherley et al. 1980; Higgins and Thorpe 1990) and is probably due to less muscle fibre hyperplasia in young stages of some fish (Kiessling et al. 1991).

In fish, somatic growth is mainly a function of skeletal muscle growth. If early posthatch growth is mainly a result of muscle fibre hypertrophy, then larvae with larger and fewer muscles fibres may have less potential for hypertrophy compared with larvae with more smaller muscle fibres. Furthermore, any muscle fibre hyperplasia and hypertrophy that may occur in posthatch fish larvae could be influenced by the density of muscle nuclei. Salmon embryos incubated at higher temperatures produce hatched larvae with a lower density of nuclei in the muscle tissue (Usher et al. 1994). In view of this profound effect of temperature on myogenesis of embryonic salmon and the correlation between fish growth and muscle fibre growth dynamics, the aim of the present study was to investigate the effect of incubating temperature on the growth and muscle cellularity of posthatch stages of Atlantic salmon.

Materials and methods

Fish

All eggs were the progeny of a single pair of Atlantic salmon (one 2+ female and one 1+ male) that originated from the river Almond. The fertilized eggs, larvae, and fry were maintained at the Almondbank salmon hatchery station, DAFFS, Freshwater Fisheries Laboratory, Pitlochry, Scotland, U.K. After fertilization, the eggs were divided into two groups; each was placed on Perspex salmon egg incubating trays kept in plastic tanks. One group was kept at the ambient river temperature of the Almondbank hatchery (where the mature salmon of the local stock would spawn). The temperature of the other group was gradually raised to 11°C over 6 days and then maintained at the temperature throughout the experiment.

Ambient temperature was considerably lower than 11°C until the hatching of the ambient incubated fish embryos, at which time the temperature gradually increased such that at first feeding the river temperature fluctuated around 10–11°C (Fig. 1).

Measurements of fish size

From each group samples were taken at hatching (when 50% of eggs hatched), first feeding, and 3 weeks after first feeding. From each group five fish per stage were over-anaesthetized in a solution of ethyl *m*-aminobenzoate methanesulfonate (MS-222). Length was measured to the nearest 1 mm. The total length was recorded on hatching, whereas for the other stages the fork length was recorded (Peterson et al. 1977). The fish were blotted dry with filter paper and the body weight, without the yolk for the hatched larvae, was recorded to the nearest 0.1 mg using a precision electronic balance (Sartorius 1712). During sampling it was observed that, at first feeding, the fish from the ambient group were similar in weight to the 11°C fish 3 weeks after first feeding. It was originally planned to take muscle samples only at hatching and at 3 weeks after first feeding for each group. However, to examine a possible correlation between fish body mass and muscle cellularity, muscle

Table 1. Wet weight (BW), length, and condition factor of developing salmon larvae.

Developmental stage	Temperature	Days from fertilization	Weight (BW) (mg) $n = 5$	Length (cm)	Condition factor (BW/L^3) ($g \cdot cm^{-1} \times 100$)	Specific daily growth rate (%)
Hatched	Ambient	117	39 (0.58)	2.0	0.48	
Hatched	11°C	40	29.14 (1.48)	1.9	0.42	
First feeding	Ambient	147	102.6 (0.24)	2.5	0.65	3.22
First feeding	11°C	80	76.6 (8.39)	2.3	0.62	3.23
weeks after first feeding	Ambient	168	204.2 (5.81)	2.9	0.83	2.40
weeks after first feeding	11°C	101	94.2 (1.13)	2.5	0.60	1.00

Note: Numbers in brackets indicate the standard error of the mean (SE). The SE of length is not given because it is less than mm which was the precision of the measurements at each stage. Specific daily growth rate was estimated using the equation $\ln(BW t_2) - \ln(BW t_1))/\text{days} \times 100$.

amples were also taken from the ambient group fish at irst feeding.

Morphometry of myofibres

The whole body posterior to the vent was mixed and mbedded according to Stickland et al. (1988). Transverse ections of 0.5-μm thickness were obtained using a Reichert ltramicrotome. The sections were treated with a saturated olution of ethanolic KOH for partial removal of resin and tained according to Ontell (1974). All measurements were nade using a computerised image analysis system (Seescan td., U.K.) linked to an Olympus (Olympus BII-2) light nicroscope. The total cross-sectional area of white muscle vas traced on the screen for one side of the transverse ection. Care was taken to exclude fin muscle fibres and the kin. The total number of white muscle fibres on this one ide of the section was counted using high magnification. The cross-sectional area of individual white muscle fibres $n > 200$) from the centre of the upper epaxial region of ach side of the myotome as used by Stickland et al. (1988) vas traced manually and recorded. The number of nuclei per ross-sectional area of white muscle was counted for each ish in five sample areas of the upper epaxial muscle, each f 6000 μm^2, using oil immersion light microscopy. The ignificance of differences in the measured parameters was etermined by Student's *t*-test between the two groups.

Results

mbryos incubated at a constant temperature of 11°C atched considerably earlier than those at ambient tem- erature (Table 1). The hatched larvae of the ambient group vere significantly ($P < 0.002$) heavier than the 11°C group.

At 3 weeks after first feeding the ambient-reared alevin ad more than twice the weight of the 11°C counterparts nd their condition factor was higher (Table 1).

On hatching, the total white muscle cross-sectional area vas not different between the two groups (Fig. 2*a*) while he myofibres of the ambient group were significantly ($P = .001$) smaller compared with the myofibres of the 11°C roup (Fig. 2*b*). Consequently, the number of myofibres vas significantly higher ($P = 0.007$) in the ambient group Fig. 2*c*).

At 3 weeks after first feeding, the total muscle cross-sectional area was larger ($P = 0.014$) in the ambient group (Fig. 2*a*), but the mean size of white muscle fibres was not significantly different between fish of the two groups (Fig. 2*b*). Moreover, there is no evidence of different rates of muscle fibre hyperplasia, as seen by muscle fibre size–frequency distributions (Fig. 3). However, the total number of myofibres was significantly higher ($P = 0.021$) in the ambient group (Fig. 2*c*).

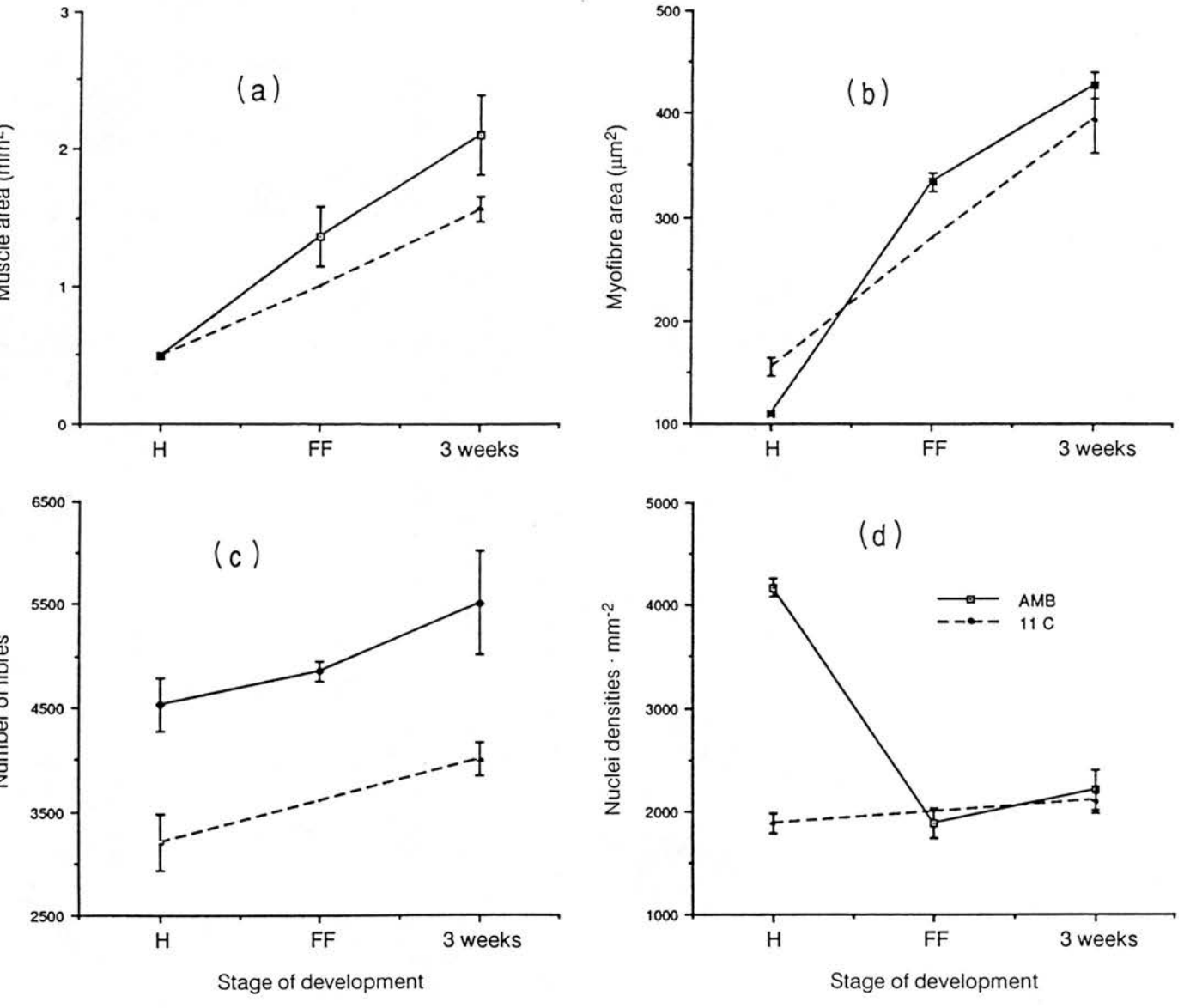
On hatching, the density of nuclei per cross-sectional area of white muscle was significantly higher ($P < 0.001$) in larvae of the ambient group, but subsequently the density of nuclei became similar in the two groups (Fig. 2*d*).

Discussion

Results indicate that incubating temperature can have a significant effect on posthatch larval growth at least up to 3 weeks after first feeding. Temperature had an accelerating effect on the growth rate of embryonic stages of development. Although the embryos incubated at a constant temperature of 11°C hatched considerably earlier than those at ambient temperature (Table 1), the hatched embryos that were incubated at the ambient temperature tended to be longer and heavier (Table 1). Peterson et al. (1977) also concluded that incubating salmon embryos at 6–7°C and then raising the temperature to 8–12°C after hatching, resulted in improved growth and larger size at first feeding than embryos and larvae reared constantly at 12°C throughout development. The relatively small difference in the size of hatched larvae became more obvious by first feeding and further increased after exogenous feeding (Table 1). This is in agreement with Beacham et al. (1985) who found, in other species of salmon, that small differences in size of embryos at hatching were enlarged by first feeding. Larval metamorphosis and transition from endogenous to exogenous feeding seems to dramatically change the body weight to length ratio in the ambient group (Table 1). The weight of the developing salmon tends to increase more rapidly than length after larval metamorphosis.

First feeding occurred at 32 days after hatching in the ambient group, whereas in the 11°C group, first feeding occurred at 40 days after hatching (Table 1). Because the

Fig. 2. Muscle growth and development of salmon reared at the ambient (solid lines) thermal regime and at 11°C (broken lines) on hatching (H), first feeding (FF), and 3 weeks after first feeding (3WKS). In each figure the bars indicate the standard error of the mean. (a) Total white muscle cross-sectional area (mm^2). (b) Mean myofibre cross-sectional area (μm^2) of white epaxial muscle. (c) Total number of white muscle fibres. (d) The density of nuclei per cross-sectional area of white muscle. Nuclei densities are adjusted for a cross-sectional area of 1 mm^2.

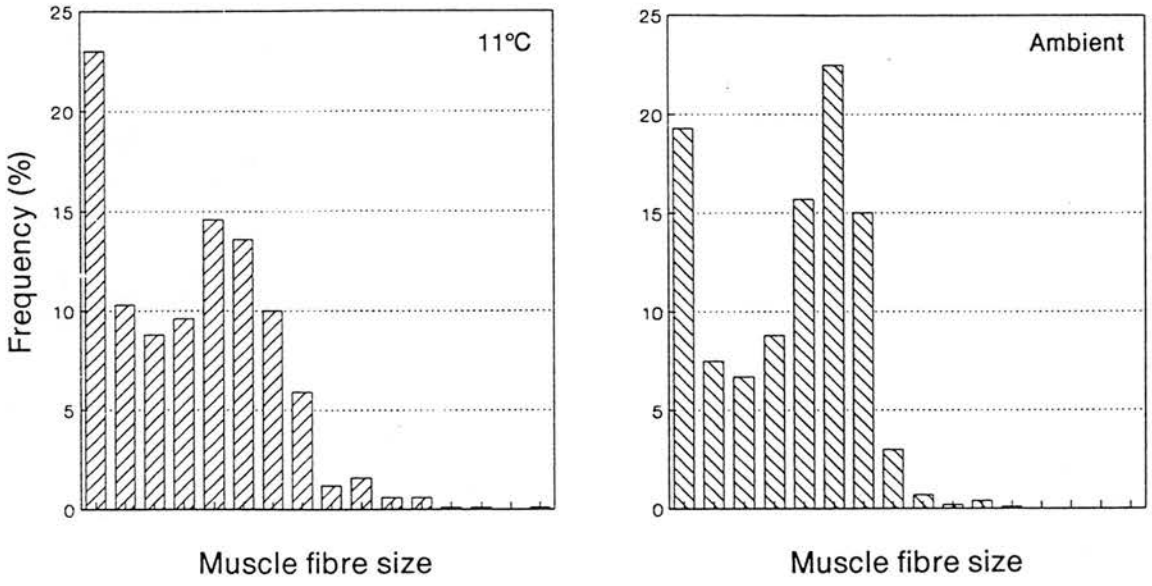


ambient temperature was gradually rising so that the mean temperature between hatching and first feeding was 9.7°C, and between first feeding and 3 weeks after first feeding it was 10.6°C, this difference in length of time between hatching and first feeding must have been a result of the incubation temperature and not of the posthatch temperature regimes. Furthermore, it should be stressed that the timing of first feeding does not coincide with the stage of 0% yolk. There is in fact a period of mixed endogenous and exogenous feeding (Kamler 1992). The faster growth and development of the ambient group probably occurred because this stock was adapted to the natural temperature regime of the river Almond. Such a growth difference indicates that the ambient thermal regime was the optimum for growth and development of the salmon used in this study.

The effect of incubation temperature on the development of posthatch salmon seen in the present study and also by Peterson et al. (1977) may be explained by the effect of temperature on muscle fibre hyperplasia and hypertrophy. On hatching there were more muscle fibres in the ambient group and the difference was similar to that found in hatched salmon incubated at ambient and 10°C (Stickland et al. 1988). At first feeding, the ambient group had more muscle fibres than the 11°C group had at 3 weeks after first feeding (Fig. 2*c*).

There is some evidence that muscle fibre hyperplasia is relatively low in young stages of development in rainbow

Fig. 3. The size–frequency distribution, expressed as percentages, of the white muscle fibres in salmon for the ambient and the 11°C group 3 weeks after first feeding. (n = 5 fish from each group).



trout (*Oncorhynchus mykiss*) (Weatherley et al. 1980; Kiessling et al. 1991) and Atlantic salmon (Higgins and Thorpe 1990). In this study also, muscle fibre hyperplasia was low in both groups (Fig. 2*c*). At hatching, larvae of the ambient group have smaller but more numerous muscle fibres and more nuclei (Figs. 2*b*–2*d*) compared with the larvae of the 11°C group. The smaller muscle fibres may exhibit a more optimum size for the diffusion of metabolites and greater potential for further growth. The relatively fewer nuclei in the 11°C larvae at hatching compared with ambient larvae may also be a contributory factor to the lower hypertrophy or hyperplasia, in that nuclei are required for both processes. As far as muscle fibre hyperplasia is concerned, more nuclei are required for the formation of new myofibres through fusion of precursor cells to form myofibres. Equally important is the number of nuclei for hypertrophic growth of muscle fibres because more nuclei are required to maintain an optimum ratio of nuclei to cytoplasm.

Salmon embryos are not active until towards the end of the endogenous feeding period and consequently muscle fibres are functionally more important after larval metamorphosis. In wild stocks, both larval growth and survival are critical for determining the recruitment in a stock, and directly affect the strength of the first year class (Gulland 1965). Larger size on first feeding can result in improved swimming performance, which will enhance active feeding (Blaxter 1969) and survival (Kazakov 1981; Wallace and Aastord 1984; Pepin and Myers 1991) of the exogenously feeding salmon larvae. This investigation has demonstrated that embryonic development of salmon at high incubation temperatures can have significant negative consequences on the muscle development, the larval size at first feeding, and consequently the viability of posthatch salmon. However, the results of this study were obtained with a Scottish Atlantic salmon stock and may not apply to other stocks of Atlantic salmon. Different stocks spawn at different seasons and environmental conditions and may, therefore, be adapted to their own particular environment. For example, there is some evidence that the effect of temperature on the rate of development of seasonally distinct spawning stocks of chum salmon (*Oncorhynchus keta*) may vary (Tallman 1986). Nevertheless, for the Atlantic salmon used in this study, a raised incubation temperature appears to have a clear effect on muscle hyperplasia and hypertrophy with consequential negative effects on posthatch growth at least up to 3 weeks after the initiation of exogenous feeding.

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References

Beacham, T.D., Withler, F.C., and Morley, R.B. 1985. Effect of egg size on incubation time and alevin fry size in chum salmon (*Oncorhynchus keta*) and coho salmon (*Oncorhynchus kisutch*). Can. J. Zool. **63**: 847–850.

Blaxter, J.H.S. 1969. Development: eggs and larvae. *In* Fish physiology Vol. 3. *Edited by* W.S. Hoar and D.J. Randal. Academic Press, London. pp. 178–241.

Farmer, G.T., Hubley, P.D., Jansen, H., McAskill, J.W., and Robin, G.B. 1990. Production of juvenile Atlantic salmon (*Salmo salar*) at the Mactaquac accelerated rearing facility, New Brunswick, Canada. Can. Tech. Rep. Fish. Aquat. Sci. 28. pp. 107–118.

Gulland, J.A. 1965. Survival of the youngest stages of fish, and it's relation to year class strength. ICNAF Spec. Publ. 6. pp. 363–371.

Higgins, P.J., and Thorpe, J.E. 1990. Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon (*Salmon salar* L.). J. Fish Biol. **37**: 505–519.

Hokanson, K.E.F., and Kleiner, C.F. 1974. Effects of constant and raising temperatures on survival and developmental rates of embryonic and larval yellow perch, *Perca flavescens* (Mitchill). *In* The early life history of fish. *Edited by* J.H.S. Blaxter. Springer-Verlag, Berlin. pp. 437–448.

Kamler, E. 1992. Early life history of fish: an energetics approach. Chapman & Hall, London. Fish and Fisheries Series 4.

Kazarov, R.V. 1981. The effect of size of Atlantic salmon (*Salmo salar* L.) eggs on embryos and alevins. J. Fish Biol. **19**: 353–360.

Kiessling, A., Storebakken, T., Asgard, T., and Kissling, K.H. 1991. Changes in the structure and function of the epaxial muscle of rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age. I. Growth dynamics. Aquaculture, **93**: 335–356.

Klinkhart, M.B., Straganov, A.A., and Pavlov, D.A. 1987. Motoricity of Atlantic salmon embryos (*Salmo salar* L.) at different temperatures. Aquaculture, **64**: 219–286.

Luczynski, M. 1985. Survival of *Coregonus albula* (L.) embryos incubated at different thermal conditions. Hydrobiologia, **121**: 51–58.

Ontell, M. 1974. Muscle satellite cells: a validated technique for light microscopic identification and a quantitative study of changes in their population following denervation. Anat. Rec. **178**: 211–228.

Pepin, P., and Myers, R.A. 1991. Significance of egg and larval size to recruitment variability of temperate marine fish. Can. J. Fish. Aquat. Sci. **48**: 1820–1828.

Peterson, R.H., Spinney, H.C.E., and Sreedharan, A. 1977. Development of Atlantic salmon (*Salmon salar* L.) eggs and alevins under varied temperature regimes. J. Fish. Res. Board Can. **34**: 31–43.

Stickland, N.C., White, R.N., Mescall, P.E., Crook, A.R., and Thorpe, J.E. 1988. The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L.) Anat. Embryol. **178**: 253–257.

Tallman, R.F. 1986. Genetic differentiation among seasonally distinct spawning populations of chum salmon (*Oncorhynchus keta*). Aquaculture, **57**: 211–217.

Usher, M.L., Stickland, N.C., and Thorpe, J.E. 1994. The role of temperature in the development of muscle cellularity in Atlantic salmon (*Salmo salar* L.) embryos. J. Fish Biol. **44**: 953–964.

Wallace, J.C., and Aastord, D. 1984. An investigation of the consequences of egg size for the culture of Arctic charr (*Salvelinus alpinus* L.). J. Fish Biol. **24**: 427–435.

Weatherley, A.H., and Gill, H.S. 1987. The biology of fish growth. Academic Press, London.

Weatherley, A.H., Gill, H.S., and Rogers, S.C. 1980. The relationship between mosaic muscle fibres and size in rainbow trout (*Salmo gairdneri*). J. Fish Biol. **17**: 603–610.

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Temperature- and developmentally-induced variation in the histochemical profile of myofibrillar ATPase activity in carp

C. NATHANAILIDES*, O. LOPEZ-ALBORS† AND N. C. STICKLAND*

**Department of Veterinary Basic Sciences, Royal Veterinary College, University of London, Royal College Street, London NW1 0TU, U.K. and †Department of Anatomy and Embryology, University of Murcia, 3004 Murcia, Spain*

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The histochemical profile of calcium activated acid stable myofibrillar ATPase (mATPase) activity in developing larval and juvenile carp was investigated. In the larval fish, differentiation of pink muscle fibres occurred after metamorphosis which was delayed by a week at 17° C compared to larvae grown at 27° C. After metamorphosis the 27° C group exhibited some small myofibres with acid stable mATPase activity in the deep white muscle. This was similar for the juvenile carp which were acclimated for more than a month at 25° C. In contrast, the cold (12° C) acclimated juvenile fish, contained very few small white muscle fibres with acid stable mATPase activity. It was also noted that the cold acclimated fish had lower background acid stable mATPase activity than the warm acclimated fish. Results indicate that after metamorphosis and more evidently in juveniles, temperature can influence the rate of myofibre hyperplasia.

Key words: *Cyprinus carpio*; temperature; metamorphosis; development; mATPase.

INTRODUCTION

Different muscle fibre types in fish are located in distinct anatomical regions, and can be characterized according to their histochemical and immunohistochemical properties (Vegetti *et al.*, 1993). Generally, newly-hatched larvae have poorly differentiated muscle fibres. As larval development proceeds, red muscle fibres differentiate and form a superficial layer which extends deeper into the myotome at the lateral line.

The ontogeny of red and white muscle fibre differentiation has been studied in a few species including carp *Cyprinus carpio* L. (Talesara & Ufri, 1987), *Brachydanio rerio* Hamilton-Buchanan (van Raamsdonk *et al.*, 1978, 1982) and sea bass *Dicentrarchus labrax* L. (Scapolo *et al.*, 1988). The differentiation of pink muscle is less rapid and is preceded by the differentiation of red and white muscle fibres in *D. labrax* (Scapolo *et al.*, 1988). Information on the development of pink muscle in carp is lacking and in fish larvae of other species is limited. The pink muscle of carp can be identified using its mATPase activity (Talesara & Ufri, 1987; Scapolo & Rowlerson, 1987). Histochemical, immunohistochemical and biochemical investigations have demonstrated a progressive appearance and disappearance of different fibre types and myosin isoforms in skeletal muscle of developing vertebrates (Lowey *et al.*, 1983; Marenchall *et al.*, 1984; Focant *et al.*, 1992) including fish such as sea bass (Scapolo *et al.*, 1988; Veggetti *et al.*, 1993) and Arctic charr *Salvelinus alpinus* L. (Martinez *et al.*,

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1991). The effect of temperature on larval stages of turbot *Scophthalmus maximus* L. is mainly on the distribution of different muscle fibre types rather than the growth of the developing larvae (Calvo & Johnston, 1992). By contrast, the rate of growth and development of carp at early larval stages can be profoundly affected by environmental temperature (Kossakoski & Jezierska, 1984). There is evidence to suggest that certain differences in the histochemical properties of mATPase can be related to differences in the myosin isoforms of carp muscle (Rowlerson *et al.*, 1985; Scapolo & Rowlerson, 1987). It is also apparent that temperature can induce changes in the myosin isoforms of carp (Gerlach *et al.*, 1990). The present study aimed to investigate whether temperature or developmentally induced changes in myosin expression is reflected in myofibrillar ATPase histochemical activity in differentiating lateral musculature of carp.

MATERIALS AND METHODS

LARVAE

Newly hatched carp larvae, which had been incubated at 20° C were divided into two groups. Thirty fish in each group were maintained in 120-l plastic tanks connected to a recirculating filtering system with a flow rate of 16 l min^{-1}. The temperature was controlled by four immersion heaters via an electronic thermostat. The tanks were aerated and the oxygen content was monitored with an oxygen probe and maintained at about 85% of saturation. The water was changed partially (by about 30%) fortnightly. A photoperiod regime of 15 h L and 9 h D was applied. Within 1 week the temperature of the two groups was gradually either raised to 27 ($\pm$ 0·1° C) or lowered to 17 ($\pm$ 0·5° C).

Fish were fed *ad libitum* with dried plankton and minced egg yolk in the first week and with a commercial fry feed thereafter. Two fish were removed every week and their external anatomical features were observed. For histochemistry, five larvae per group were taken at the fourth, fifth and sixth week after hatching. Fish were anaesthetized with ethyl m-aminobenzoate methasulfonate (Sigma, U.S.A.) according to Summerfelt & Smith (1990) and decapitated. The whole larval body was coated in embedding cryo medium (BDH, U.K.) and frozen in isopentane cooled close to its freezing point in liquid nitrogen, and frozen transverse sections posterior to the vent were obtained for histochemistry.

JUVENILES

Laboratory reared carp juveniles (15·62 g $\pm$ 0·59), originally kept at ambient temperature, about 5 months old were kept for at least 5 weeks at either 12 ($\pm$ 1·5° C) or at 25 ($\pm$ 1·5° C). Each group was kept in tanks of 120 l supplied with dechlorinated tap water heated with thermostatically controlled immersion heaters. Frozen transverse sections of the deep white lateral musculature of three fish per group were taken just caudal to the vent.

MYOFIBRILLAR ATPase

In preliminary studies it was observed that the carp pink muscle was readily identified using the method of Mascarello *et al.* (1984) after preincubation in a solution of 0·05 M sodium acetate at a pH of 4·40–4·60 or after alkaline preincubation (pH 10·1–10·3), with acid preincubation resulting in a higher contrast of pink muscle than the alkaline. The existence of pink muscle was also verified according to its intermediate activity of succinate dehydrogenase (Carpene *et al.*, 1982) using the method of Dubowitz & Brooke (1973).

TABLE I. Development of carp larvae reared at 17 and 27° C

Week	Stage	
	27° C	17° C
1	B	B
2	C1-2	C1
3	D2	D1
4	E	D2
5	F	E
6	Fry	F

Developmental stages B, C, D, E and F according to Kossakoski & Jezierska (1984), stage F is the final stage of metamorphosis.

MUSCLE MORPHOMETRY AND GROWTH DYNAMICS

Sections were also stained with haematoxylin and eosin for morphometric analysis. The areas of at least 150 muscle fibres from sectioned areas of the deep white muscle in the upper epaxial region, were measured using a computerised image analysis system (Seescan plc, Cambridge, U.K.). The ratio of mean myofibre size to fish size, which is inversely correlated with muscle fibre hyperplasia in young fish (Weatherley *et al.*, 1979), and myofibre size frequency distributions were used in order to assess the relative rate of hyperplasia and hypertrophy of myofibres. The significance of differences was determined using a Student's t test ($P<0{\cdot}05$).

The theoretical total number of myofibres in the region posterior to the vent was estimated according to Higgins & Thorpe (1992) by dividing the total white muscle cross sectional area (of one side of the fish) with the mean myofibre size. The theoretical number of myofibres was not estimated in the juveniles because complete sections could not be obtained due to the relatively large size.

RESULTS

LARVAE

The stages of larval development were based on the development of exoskeleton and pigmentation according to Kossakoski & Jezierska (1984). The ratios of mean myofibre size to body weight of 6-week-old fish grown at 17 and 27° C were not significantly different (Table I). Also, the average daily growth rates over the period of this study were not significantly different (Table II).

The mATPase activity of all muscle fibres was labile to acid preincubation at all pHs used up to the third week after hatching. On the fourth week the fish of the 27° C group exhibited a sparse monolayer of some intermediate fibres, which were concluded to be pink, between the red and white muscle fibres, with acid stable and alkaline stable (not illustrated) mATPase activity. By the fifth week, the 27° C group had a continuous layer of pink muscle fibres with acid stable mATPase activity [Fig. 1(a)] whereas the 17° C group had a sparse layer of muscle fibres with acid stable mATPase activity [Fig. 1(b)], resembling the situation seen in the 27° C fish a week earlier. At the sixth week, the pink fibres in the 27° C group formed a thicker layer compared to the 17° C group [Fig 1(c), (d)]. Furthermore, the white muscle of the 27° C fish exhibited a

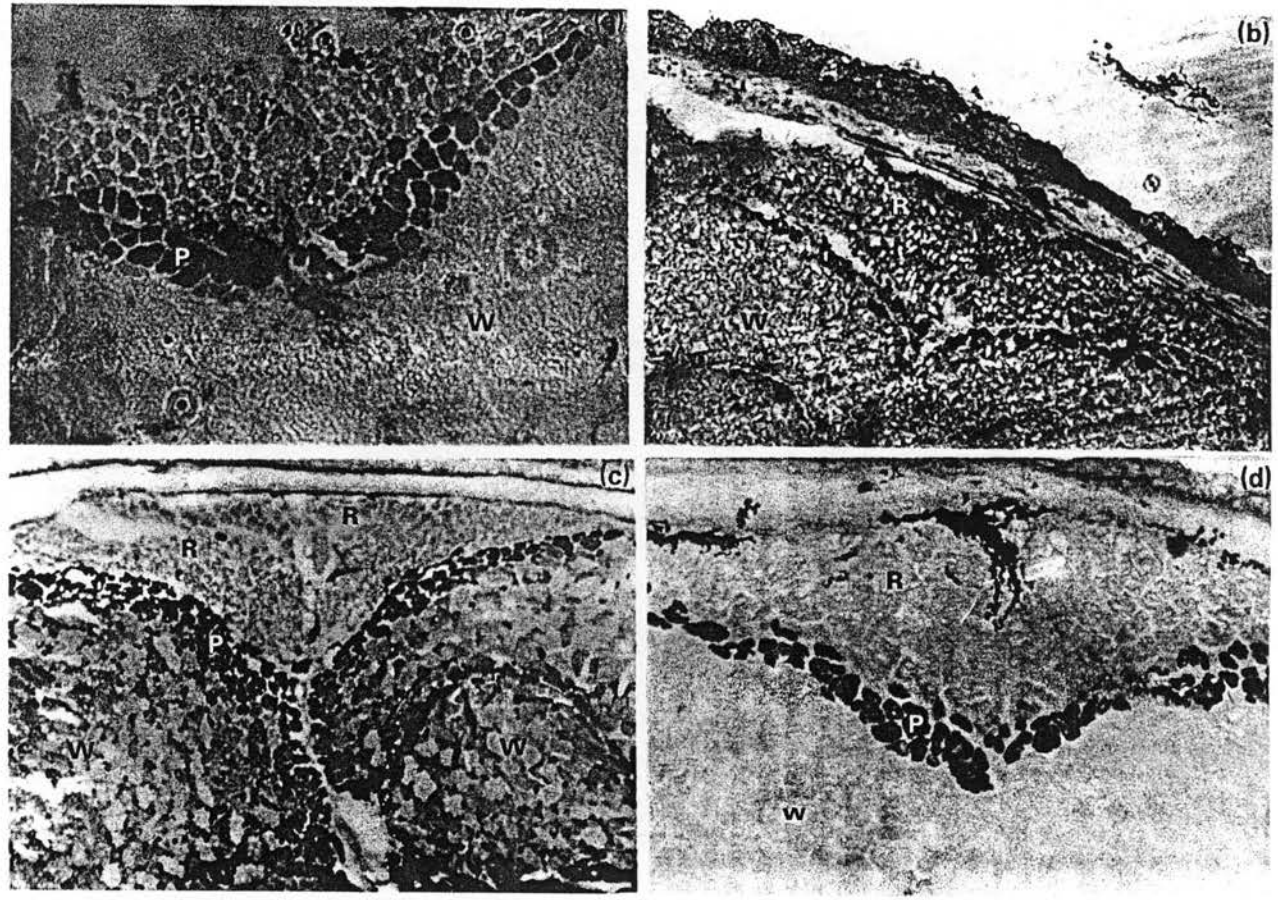

FIG. 1. mATPase activity after acid pre-incubation (pH 4·6 for 30 s) in 5-week-old post-hatch carp larvae reared at 27° C (a) and at 17° C (b), and in 6-week-old carp larvae reared at 27° C (c) and at 17° C (d). × 42. Labels R, P and W indicate the red, pink and white muscle fibres respectively.

TABLE II. The ratio of mean myofibre size (mm^2) to body weight (g) of growing carp ($n=3$)

	SGR%	Ratio	No. of myofibres
6 weeks			
17° C	1·78	$0{\cdot}39 \times 10^{-3}$ (0·06)	1745·58 (148·52)
27° C	2·16	$0{\cdot}25 \times 10^{-3}$ (0·04)	2259·67 (108·20)
	NS	NS	$P<0{\cdot}05$
5 months			
12° C	0·80	$0{\cdot}180 \times 10^{-3}$ (0·06)	
25° C	2·61	$0{\cdot}089 \times 10^{-3}$ (0·01)	
	$P<0{\cdot}01$	$P<0{\cdot}01$	

The daily specific growth rate (SGR%, $n=5$) over the period of this study was estimated according to the equation: $\ln W_2 - \ln W_1$/no. Days. (W_2 and W_1 are body weight in the end and the start of the experiment respectively.) Numbers in parentheses indicate the S.E.M. (NS, not significant).

diversity in mATPase activity. Small fibres with acid stable mATPase activity were found within the white musculature [Fig. 1(c)]. Frequency histograms of white muscle fibre size indicated the existence of more small myofibres in the 27° C group (Fig. 3). This can be seen in the greater number of fibres in the smallest size class at 27° C. Nevertheless there were also larger muscle fibres in the 27° C group which must have significantly influenced the overall muscle growth of the 27° C fish. As a result, in spite of the difference in the size–frequency distribution of small myofibres, the ratio of mean myofibre size to body weight of the 27° C group was not significantly different from that of the 17° C fish (Table II). In contrast, the theoretical number of myofibres was significantly higher in the 6-week fish of the 27° C group compared to the 17° C fish of the same age (Table II).

JUVENILES

Carp acclimated at 25° C exhibited heterogeneous muscle fibres in terms of mATPase activity, with small white muscle fibres having an acid stable mATPase activity [Fig. 2(a)]. On the other hand, all white muscle fibres of the cold acclimated carp were largely homogeneous in terms of mATPase activity which was acid labile irrespective of the muscle fibre size with very few exceptions [Fig. 2(b)]. The overall mATPase activity was higher in the large white muscle fibres in the 25° C acclimated carp, compared to the 12° C group (Fig. 2). Frequency histograms of white muscle fibre size indicated a higher level of hyperplasia in the 25° C group (Fig. 3). Moreover, the 25° C juveniles exhibited a higher specific growth rate and a lower mean myofibre size to body weight ratio than the 12° C group (Table II).

DISCUSSION

In the larvae reared at 27° C, a continuous layer of pink muscle fibres appeared at the fifth week and developed further at the sixth week, whereas at 17° C, the larvae exhibited only a sparse layer of pink muscle at the fifth week (Fig. 1). In

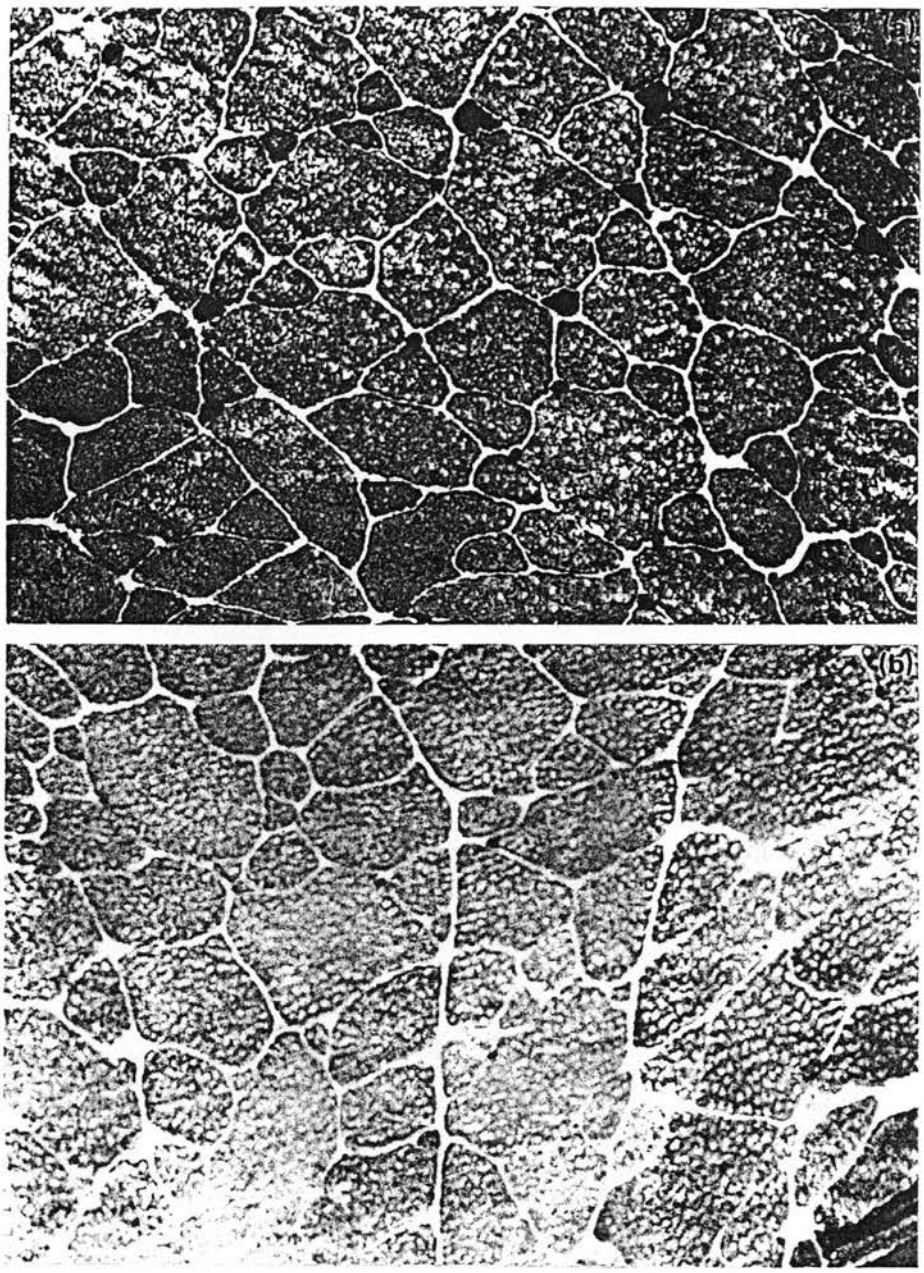

FIG. 2. mATPase activity after acid pre-incubation (pH 4·6) in juvenile carp larvae acclimated for 6 weeks at 25° C (a) and at 12° C (b). × 59.

sea bass the pink muscle is not seen before about 60 days post-hatching (Scapolo *et al.*, 1988). Waterman (1969) suggested that the pink muscle fibres of *Brachydanio rerio* may originate from differentiating white muscle fibres. It has been demonstrated in several species that the muscle differentiation can be related to swimming behaviour of the developing fish (Nag & Nursall, 1972; van

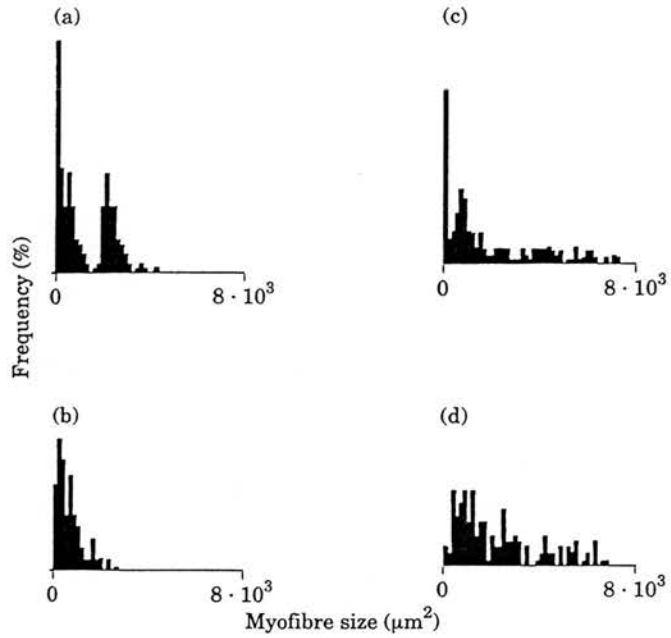

FIG. 3. The frequency distribution of white muscle fibres in 6-week-old post-hatch carp reared at 27° C (a) and at 17° C (b), and in juvenile carp reared for 6 weeks at 25° C (c) and at 12° C (d).

Raamsdonk *et al.*, 1978; Proctor *et al.*, 1980; Matsuoka & Iwai, 1984; Scapolo *et al.*, 1988). In the present study the colder temperature delayed the final larval metamorphosis, as has been shown by Kossakoski & Jezierska (1984). Nevertheless, in both temperature groups, the emergence of pink muscle fibres coincided with the final stage of larval metamorphosis (Table I). A well-developed layer of pink muscle fibres appeared, in both temperatures, when larvae had fully formed scales and functional fins (Table I and Fig. 1). This indicates that carp pink muscle, which is recruited at intermediate swimming speeds (Johnston *et al.*, 1977), may be of functional significance for post larval stages, which are characterized by a different mode of swimming compared to the larval fish (Batty, 1984). In the same manner sea bass pink muscle formation occurs when swimming behaviour, performance and habitats are characteristic of juvenile stages (Scapolo *et al.*, 1988). Small white muscle fibres of carp with strong mATPase activity after acid preincubation observed in this study (Fig. 1) have been found to have different immunohistochemical properties from the large mature white muscle fibres (Rowlerson *et al.*, 1985; Scapolo & Rowlerson, 1987). Small white fibres of carp exhibit a cross reaction with both anti-slow and anti-fast serum indicating either a mixture of myosin isoforms or an embryonic type of myosin (Rowlerson *et al.*, 1985). Small white muscle fibres which differ in histochemical mATPase activity have also been reported in another cyprinid, *Chalcalburus chalcoides* Güldenstädt (Sanger, 1992) as well as in *Mugil saliens* Risso (Carpene *et al.*, 1982). If it is assumed that the very small muscle fibres are newly formed fibres (Weatherley & Gill, 1981; Stickland, 1983), and that the ratio of mean myofibre size is inversely related with the rate of muscle fibre hyperplasia (Weatherley *et al.*, 1979) it would seem that muscle fibre hyperplasia

is increased in the fast growing warm acclimated juvenile carp (Table II). In the same manner, the occurrence of ' new ' small muscle fibres in the grey mullet can also be linked to elevated seasonal temperatures associated with fast growth rates (Carpene *et al.*, 1982). By contrast, in the 6-week-old fish the ratio of mean myofibre size to body weight was not significantly affected by temperature (Table II). This is because muscle hyperplasia has just started at the sixth week in the 27° C fish [Fig. 1(a), (c)]. This is also demonstrated by the percentage of the smaller size class in the size–frequency histograms [Fig. 3(a), (b)] of myofibres which also show a wider distribution of myofibres in the 27° C fish. As a result although the theoretical number of myofibres was higher in the 27° C group, the main effect of temperature on larval stages appears to be in the distribution and differentiation of muscle fibres rather than the rate of growth (Table II). This is in agreement with Calvo & Johnston (1992) who also reported similar results with developing turbot.

There is some evidence to suggest that, in carp, different myosin isoforms are present at cold and warm temperatures (Gerlach *et al.*, 1990) but it is unclear whether such a difference is due to a different isoform found in the mature white muscle fibres at warm temperature, or to the higher number of small muscle fibres, with a different myosin isoform. The variation in the overall stability of the histochemical mATPase activity, in the white muscle fibres between the two temperatures may be due to different myosin isoform proportions present at different temperatures for all fibres as well as the difference for the small fibres already discussed.

In conclusion, the results have shown that temperature can affect the timing of differentiation of pink muscle which coincides with the timing of metamorphosis. Temperature also appears to affect the profile of acid stable mATPase histochemical activity in the white muscle of carp.

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References

Batty, R. S. (1984). Development of swimming movements and musculature of larval herring *Clupea harengus*. *Journal of Experimental Biology* **110,** 217–229.

Calvo J. & Johnston, I. A. (1992). Influence of rearing temperature on the distribution of muscle fibre types in the turbot *Scophthalmus maximus* at metamorphosis. *Journal of Experimental Marine Biolology* **161,** 45–55.

Carpene, E., Veggetti, A. & Mascarello, F. (1982). Histochemical fibre types in the lateral muscle of fishes in fresh, brackish and salt water. *Journal of Fish Biology* **20,** 379–396.

Dubowitz, V. & Brook, M. H. (1973). *Muscle Biopsy: A Modern Approach*. London: W. B. Saunders.

Gerlach, G. F., Turay, L., Malic, K. T., Lida, J., Scutt, A. & Goldspink, G. (1990). Mechanisms of temperature acclimation in the carp: a molecular biology approach. *American Journal of Physiology* **259,** R237–R244.

Higgins, P. J. & Thorpe, J. E. (1992). Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology* **37,** 505–519.

Focant, B., Huriax, F., Vandewalle, P., Castelli, M., & Goessens, G. (1992). Myosin, paralbumin and myofibril expression in barbel (*Barbus barbus* L.) lateral white muscle during development. *Fish Physiology and Biochemistry* **10,** 133–143.

Johnston, I. A., Davison, W. and Golspink, G. (1977). Energy metabolism of carp swimming muscles. *Journal of Comparative Physiology* **114B,** 203–216.

Kossakoski, M. K & Jezierska, B. (1984). The influence of thermal conditions on postembryonic development of some species of Crenidae and Cyprinidae. *Zoologica Poloniae* **31,** 43–56.

Lowey, S., Benfield, P. A., Leblanc, D. D. & Waller, G. S. (1983). Myosin isoenzymes in avian skeletal muscles. I. Sequential expression of myosin isoenzymes in developing chicken pectoralis muscle. *Journal of Muscle Research and Cell Motility* **4,** 695–716.

Marenchall, G., Schwart, K., Beckers-Bleux, G. & Ghins, E. (1984). Isoenzymes of myosin in growing and regenerating rat muscles. *European Journal of Biochemistry* **138,** 421–428.

Martinez, I., Christiansen, J. S., Ofstad, R. & Olsen, R. L. (1991). Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Arctic charr (*Salvelinus alpinus* L.). *European Journal of Biochemistry* **195,** 743–753.

Martinez, I. & Pettersen, G. W. (1992). Temperature induced precocious transitions of myosin heavy chain isoforms in the white muscle of the Arctic charr (*Salvelinus alpinus* L.). *Basic and Applied Myology* **2,** 89–95.

Mascarello, F., Romanello, M. G., & Scapolo, P. A. (1984). Histochemical and immunohistochemical profile of pink muscle fibres in some teleosts. *Histochemistry* **84,** 251–255.

Matsuoka, M. & Iwai, T. (1984). Development of the myotomal musculature in the red sea bream. *Bulletin of the Japanese Society of Scientific Fisheries* **50,** 29–35.

Nag, A. C. & Nursall, J. R. (1972). Histogenesis of white and red muscle fibres of trunk muscles of a fish *Salmo gairdneri*. *Cytobios* **6,** 227–246.

Proctor, C., Mosse, P. R. L. & Hudson R. C. L. (1980). A histochemical and ultrastructural study of the development of the propulsive musculature of the brown trout, *Salmo trutta* L. in relation to its swimming behaviour. *Journal of Fish Biology* **16,** 309–329.

Rowlerson, A., Scapolo, P. A., Mascarello, F., Carpene, E. & Veggetti, A. (1985). Comparative study of myosin present in the lateral muscle of some fish: species variations in myosin isoforms and their distribution in red, pink and white muscle. *Journal of Muscle Research and Cell Motility* **6,** 601–640.

Van Raamsdonk, W., Pool, C. W. & te Kronnie, G. (1978). Differentiation of muscle fibre types in the teleost *Brachydanio rerio*. *Anatomy and Embryology* **153,** 137–155.

Van Raamsdonk, W., van't Veer, L., Veeken K., Heyting, C. & Pool, C. W. (1982). Differentiation of muscle fibre types in the teleost *Brachydanio rerio*, the zebra fish: posthatching development. *Anatomy and Embryology* **164,** 51–62.

Sanger, A. M. (1992). Effects of training on axial muscle of two cyprinid species: *Chondrostoma nasus* (L.) and *Leuciscus cephalus* (L.). *Journal of Fish Biology* **40,** 637–646.

Scapolo, P. A. & Rowlerson, A. (1987). Pink lateral muscle in carp (*Cyprinus carpio* L.): histochemical properties and myosin composition. *Experientia* **43,** 384–386.

Scapolo, P. A., Veggetti, A., Mascarello, F. & Romanello, M. G. (1988). Developmental transitions of myosin isoforms and organisation of the lateral muscle in the teleost *Dicentrarchus labrax*. *Anatomy and Embryology* **178,** 287–295.

Stickland, N. C. (1983). Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*). *Journal of Anatomy* **137,** 323–333.

Summerfelt, R. C. & Smith, L. S. 1990. Anasthesia, surgery and related techniques. In *Methods for Fish Biology* (Schreck, C. B. & Moyle, P. B., eds), pp. 213–272. Bethesda, MD: American Fisheries Society.

Talesara, C. L. & Ufri, A. J. (1987). A histophysiological study of muscle fibre differentiation and growth in the common carp, *Cyprinus carpio* L. *Journal of Fish Biology* **31,** 45–54.

Veggetti, A., Mascarello, F., Scapolo, P. A., Rowlerson, A., Candia Carnevali, M. D. (1993). Muscle growth and myosin isoform transitions during development of a small teleost fish, *Poecilia reticulata* (Peters): a histochemical, immunohistochemical, ultrastructural and morphometric study. *Anatomy and Embryology* **187,** 353–361.

Waterman, R. E. (1969). Development of the lateral musculature in the teleost, *Brachydanio rerio*: a fine structural study. *American Journal of Anatomy* **125,** 457–494.

Weatherley, A. H., Gill, H. S. & Rogers, S. C. (1979). Growth dynamics of muscle fibres, dry weight and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). *Canadian Journal of Zoology* **57,** 2385–2392.

Weatherley, A. H. & Gill, H. S. (1981). Characteristics of mosaic muscle growth in rainbow trout *Salmo gairdneri*. *Experientia* **37,** 1102–1103.

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Is physiological hypoxia the driving force behind temperature effects on muscle development in embryonic Atlantic salmon (*Salmo salar* L.)?

Tim W. Matschak, Neil C. Stickland, Andrew R. Crook, Tanya Hopcroft

Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, UK

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Abstract. Atlantic salmon embryos raised at a higher temperature than normal exhibit, in addition to accelerated growth and development, proportionately less muscle fibre hyperplasia and proportionately more fibre hypertrophy in their presumptive white muscle tissue. The egg capsule combined with the perivitelline fluid represents an oxygen barrier and may contain metabolic by-products within the egg. The effect of removing this barrier, and thus oxygen restriction, on the development of muscle cellularity in embryonic salmon was therefore investigated in this study. It was found that the presence of the chorion has a distinct effect. Fibre hyperplasia was found to be influenced by temperature only in the presence of the egg capsule when total fibre numbers were 15% higher at 6.5°C than at 11°C. Fibre hypertrophy was increased at the higher temperature in the chorionated embryos leading to the average white fibre cross-sectional area being approximately 30% bigger. The opposite effect was found in dechorionated embryos which showed a bigger average white fibre cross-sectional area by approximately 30% at the lower temperature. These differences in the effect of temperature on muscle cellularity in embryonic Atlantic salmon grown within or without the chorion may be explained by a higher oxygen demand combined with restricted oxygen availability at the higher temperature. The difference may thus be due to physiological hypoxia at increased temperatures. This is supported by findings on the immediate posthatch growth when the restriction on fibre hyperplasia at the higher temperature appeared to be removed. Total white muscle cross-sectional areas and fish lengths were analysed as general growth parameters.

Introduction

During embryonic development and growth in fish, as in other vertebrates, muscle size increases as a result of two fundamental mechanisms. A combination of muscle fibre hyperplasia (the recruitment of new muscle fibres) and fibre hypertrophy (the increase in size of existing fibres) takes place. Whereas the former ceases to play a role in mammals around the time of birth and postnatal growth is achieved by an increase in fibre size (see for example [21]), both processes are of importance in fish throughout a substantial part of the animals' lives [23, 29].

In Atlantic salmon embryos these processes have been shown to be finely balanced in response to environmental temperature. Embryos reared at different temperatures show no difference in muscle cellularity at early developmental stages. However, in the later stages up to hatching, a proportionate increase in fibre hypertrophy and reduction in fibre hyperplasia at increased temperatures is observed [24, 26]. This is associated with accelerated growth and development at the higher temperature.

In post-hatch fish an entirely different situation is found. Faster growth of Atlantic salmon juveniles is achieved by the addition of new muscle fibres [12]. In fact, a correlation between faster growth and fibre hyperplasia has been shown both interspecifically [28] and intraspecifically (e.g. yearling rainbow trout: [30]; larval herring: [27]).

One obvious difference between the prehatch and the posthatch environment is that embryos are surrounded by an egg capsule, or chorion, and perivitelline fluid. This barrier has been shown to reduce oxygen availability to the embryos (salmon: [10]; trout: [18]). Additionally, oxygen requirements in salmonid embryos are high [20, 21]. Growth at increased temperatures leads to higher oxygen requirements [19] and salmon embryos show a substantial increase in metabolic rate with temperature [8]. Atlantic salmon eggs incubated at higher temperatures may therefore be subject to physiological hypoxia.

Energy levels have been shown to influence cell size and nuclear numbers in the muscle of rats. Energy restriction with adequate protein supply reduces nuclear numbers with normal to increased cell size suggesting a

Correspondence to: T.W. Matschak

link between calorie intake and nuclear proliferation (reviewed in [2]). It seems possible therefore that reduced oxygen availability, and thus a reduction in metabolic energy, can influence the development of muscle cellularity in the salmon.

In this study the hypothesis that muscle cellularity can be affected by the presence of the chorion was tested by removing the egg capsule. This removal increases the accessibility of oxygen to the Atlantic salmon embryos and may thus suggest the involvement of oxygen levels. The effect on the muscle cellularity of the embryos was analysed.

Methods

Animals. Salmon eggs were derived from a single adult pairing and were of a similar size. The eggs were incubated at the SOAFD Freshwater Fisheries Laboratory, Almondbank, Perthshire, Scotland at ambient river temperature (with an average of 3.4° C) until development stage 21 (after Gorodilov [4]). The staging includes a number of morphological parameters such as fin development, vascular and muscular development and some organ development. It was devised from samples taken at different temperature regimes between 0.5 and 11°C [4]. The absence of heterochronism due to different incubation temperatures for the parameters used has been shown for the genus *Salmo* [5]. At stage 21 the eggs were sent to London and kept in cooled incubators (LMS; Sevenoaks) at 6.5°C until stage 25 was reached. Muscle cellularity parameters had previously been found not to differ between incubation temperatures until after this stage [24, 26]. The egg capsule was removed mechanically (dechorionation) and the embryos were transferred into physiological saline (Hank's Balanced Salt Solution, Gibco) in 30-mm Petri dishes (Flow Laboratories). Further incubation took place in cooled incubators at 6.5°C or 11°C. Samples were taken at development stages 31 and 33. Embryos with the capsule intact were also grown as controls. Hatching took place at development stage 30 at both incubation temperatures.

Sample preparation The chorion was removed where appropriate and the embryos killed by decapitation. The head and the yolk sac were removed and the remaining tissue fixed in 4% paraformaldehyde in phosphate buffered saline (Sigma) for 1–2 h. After fixation the tissue was dehydrated in ethanol and then embedded in methacrylate employing the following method:

The tissue was infiltrated with solution A (160 ml 2-hydroxyethyl methacrylate, 32 ml 2-butoxyethanol, 2 g benzoyl peroxide) for a total of 3 h. The solution was changed every hour. Embedding mixture was prepared from 30 ml solution A and 1 ml solution B (15 ml polyethylene glycol 200, 1 ml *N,N*-dimethylaniline). The mixture was immediately poured into plastic moulds, the tissue added and oriented, and plastic or metal chucks lowered onto the moulds. The edges were sealed with paraffin and the mixture left to polymerise overnight. Finally the polymerised blocks were removed from the moulds and left to dry at room temperature for 1 day.

Transverse sections in the region of the vent were taken at a thickness of 10 µm using a glass knife on a Reichert-Jung microtome (model 1140/Autocut). The sections were stained by the method of Verhoeff and van Giesson.

Analysis. The stained sections were analysed using a Seescan image analyser connected to an Olympus research microscope. Mean white fibre cross-sectional area, total white muscle cross-sectional area and total white muscle fibre number were determined. The contribution of presumptive red muscle was ignored because it consists of only a single layer of fibres at the stages analysed.

Fig. 1 Sample cross-section through an embryonic salmon at stage 31 in the region of the vent. White muscle fibre cross-sectional area was measured within the *boxed area; NC,* notochord; *NT,* neural tube; *scale bar,* 200 µm

For mean white muscle fibre cross-sectional area a minimum of 50 fibres were analysed for each section in the top quadrant of the fish in the area indicated in Fig. 1. Total white muscle cross-sectional area and total white fibre number were determined from one lateral half of each fish. Five fish were analysed for each sampling point and treatment group. The length of 10 embryos was determined for each sampling point and treatment group from photographs taken just before sampling.

Statistics. Analysis of Variance and Newman-Keuls multiple comparisons were employed with a significance level of $P<0.05$.

Results

Embryonic development

The embryos grown without the egg capsule developed well and normally as judged by visual examination and staging, and by comparison with the development of controls incubated with the egg capsule intact.

There was no obvious difference in the rate of development of embryos grown within or without the egg

Table 1

tage	25	31		33	
ength of embryos (mm)					
Chorion	+[a]	+	–[b]	+	–
.5°C	11.85	16.62	18.85	21.39	23.58
	±0.54	±0.56	±0.49	±0.58[c]	±0.56
1°C	N/A	17.57	18.29	20.79	21.47
		±0.50	±0.34	±0.63	±0.79[c]
Development time after stage 25 (days)					
.5°C	0	25	23	36	34
1°C	0	14	13	20	19

engths of embryos and development times. All lengths apart rom the ones marked [c] showed a statistically significant differnce according to a Newmann-Keuls multiple comparison with a ignificance level of $P<0.05$, $n=10$. The standard deviation is givn. Development times are given after stage 25, the development tage at which the fish were transferred to different temperatures [a] +, present [b] –, absent

apsule. However, it was found that the embryos develped much faster once they were incubated at the higher emperature (Table 1). The development rate between tages 25 and 33 of fish transferred to 11°C at stage 25 id not appear to differ from that of eggs incubated at 1°C from fertilisation (previous season's data; data not hown).

ength

The lengths of the embryos are shown in Table 1. With ne exception (stage 31, egg capsule present) an increase n temperature led to a reduction in length. Growth of ne embryos without the egg capsule appeared to result n an increased length.

Vhite muscle cellularity

The parameters analysed were mean muscle fibre cross-ectional area as a measure for hypertrophic growth (Fig.), total fibre number as a measure for hyperplastic rowth (Fig. 3) and total white muscle cross-sectional rea as a measure for total muscle growth (Fig. 4).

tage 31. Under the conditions chosen, the encapsulated mbryos hatched at stage 30. The samples taken at stage 1 therefore represent the situation in newly hatched Atntic salmon. The data are summarised in Figs 2–4. As n previous experiments [24, 26] an increase in temperare led to a larger mean white muscle fibre cross-sec-onal area and a smaller total number of white muscle bres for encapsulated embryos. In the dechorionated mbryos the finding for mean white fibre cross-sectional rea was reversed and no statistically significant differnce in fibre number was found. Hyperplastic growth as therefore not compromised by temperature in the echorionated embryos at this stage. They achieved the same fibre numbers as the low-temperature chorionated group. Embryos grown at the higher temperature without the egg capsule reached the same fibre cross-sectional area as their lower-temperature chorionated counterparts, but the lower-temperature dechorionated group ex-

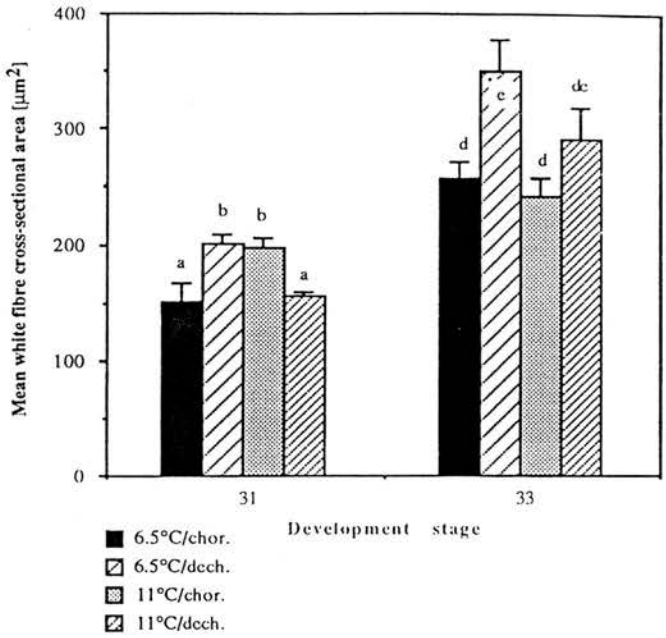

Fig. 2 Mean white muscle fibre cross-sectional area in µm² at stages 31 and 33; chor., chorionated; dech., dechorionated. The number of embryos per sample was 5. *Error bars* are s.e.m. Statistically significant differences within a development stage are indicated by *lower-case letters* ($P<0.05$)

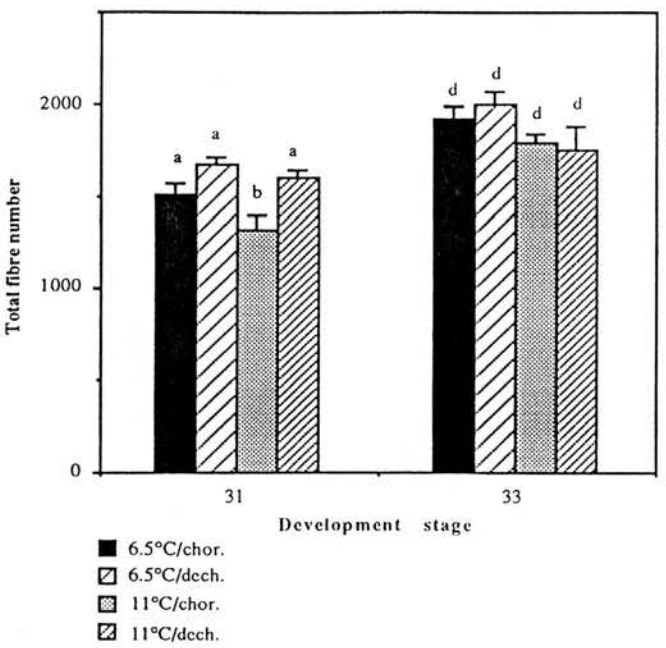

Fig. 3 Total white muscle fibre number at stages 31 and 33 determined in one lateral half of the fish. Five embryos were analysed per group. *Error bars* are s.e.m. Statistically significant differences within each development stage are indicated by *lower-case letters* ($P<0.05$)

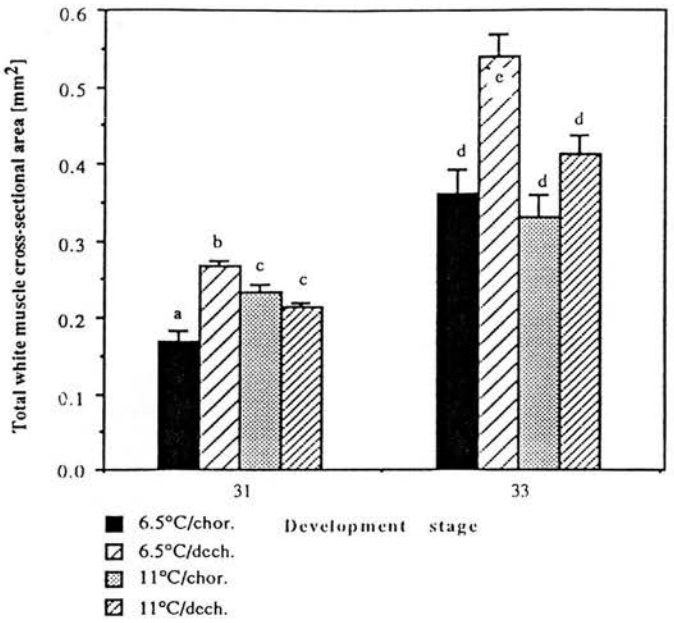

Fig. 4 Total white muscle cross-sectional area in mm² at stages 31 and 33 determined in one lateral half of the fish. Five embryos were analysed per group. *Error bars* are s.e.m. Statistically significant differences within a development stage are indicated by *lower-case letters* ($P<0.05$)

hibited increased hypertrophic growth in comparison. The total white muscle cross-sectional area was again affected differently by temperature depending on the presence of the egg capsule. The 6.5°C dechorionated group exhibited the largest and the 6.5°C chorionated group the smallest total white muscle cross-sectional area with both higher temperature groups being intermediate.

Stage 33. The samples taken at stage 33 represent the immediate post-hatch growth of the salmon. The data are shown in Figs 2, 3 and 4. No statistically significant difference in total white muscle fibre number was found at this stage. Hypertrophic growth as represented by the mean white muscle fibre cross-sectional area seems to have proceeded at a similar rate in all groups with the exception of the 11°C chorionated group. In the latter case a similar fibre cross-sectional area to the low temperature chorionated group was found. This indicates increased hyperplasia and reduced hypertrophic growth of the 11°C chorionated group in comparison with the other treatments. The total muscle cross-sectional areas were found to have increased in all groups with the lower temperature groups showing a slightly larger increase. The 6.5°C chorionated group thus reached a total muscle cross-sectional area similar to that of the 11°C groups.

Discussion

Length

The shortening generally found at the increased temperature may be due to an effect on the number of vertebrae formed. Such an effect has been found in Atlantic salmon with increased temperature leading to a reduced number of vertebrae [16]. In one group (chorionated, shortly after hatching; stage 31) this was not the case and the embryos were shorter at the lower incubation temperature. However, this may be due to an additional effect on the length of segments. The apparent difference between this study (stage 31; chorionated) and a study by Hamor and Garside [7], who found a relatively reduced weight of embryos grown at a higher temperature at "an advanced prehatching state", may be due to differences in staging. The latter authors used criteria originally developed for lake trout (*Salvelinus namaycush* Walbaum) with the stage used for analysis being defined only by the appearance of melanophores [3]. The present study employed the criteria defined by Gorodilov [4] in which the appearance of melanophores is only one of a number of factors. Additionally, the staging system used in this study has been shown to be suitable for the application to different incubation temperatures [4, 5].

The fact that the fish grown in the absence of the egg capsule achieved a greater length than the chorionated groups suggests an effect of the capsule as a physical barrier. This is supported by reports on the influence of egg size on embryo size. It is known that in Atlantic salmon larger egg size leads to an increased larval size at first feeding. Furthermore egg size does not affect the incubation time at a given temperature [13, 25]. Even though this is only partially true at hatching, it was found that egg size is positively correlated with embryo weight when there are large differences (egg weight differing by more than 20–40 mg; [13]). Again there was no difference in incubation time at a given temperature and an influence of the amount of yolk available on growth is therefore unlikely.

White muscle cellularity in newly hatched fish

The incubation of salmon eggs at different temperatures has a distinct effect on white muscle cellularity. As was found by Stickland et al. [24] and Usher et al. [26], increased temperature leads to a proportionate increase in growth by fibre hypertrophy as opposed to muscle fibre hyperplasia. The findings of this study corroborate this.

However, as this study shows, a different response occurs when the embryos are grown without the egg capsule. In this instance higher temperature resulted in proportionately reduced fibre hypertrophy but there was no effect on muscle fibre hyperplasia.

This difference in temperature response, depending on the presence or absence of the chorion, may be due to a number of factors. With the removal of the chorion and the perivitelline fluid, factors contained in the perivitelline fluid such as hormones and growth factors are lost. However, one such factor, thyroid hormone, appears to be preferentially stored within the yolk [15]. Another possible influence may be the accumulation of metabolic by-products within the chorion.

A large number of supportive evidence suggests differences in oxygen and therefore energy availability as a

decisive factor. Hamor and Garside [7] find an increase in yolk conversion efficiency at 100% air saturation after hatching and speculate that this may be related to improved oxygen availability. The oxygen availability to fish embryos does indeed increase with hatching. In rainbow trout *(Oncorhynchus mykiss)* the critical oxygen tension, that is the point at which oxygen becomes rate limiting, drops dramatically at hatching [18]. A similar effect has also been found in artificially hatched Atlantic salmon [10]. The oxygen availability to the groups grown without the egg capsule was therefore higher than to their encapsulated counterparts. There is evidence that the chorion itself consumes some oxygen [6]. However, this effect is probably small in comparison with the increase in surface:volume ratio upon removal of the chorion.

An equally important point is that oxygen requirements and metabolic rate are linked with temperature. In salmon embryos a Q_{10} value of 3.67 between 5° C and 10° C can be calculated (from hourly oxygen consumption per ovum in [8]). Due to this influence on metabolic rate, temperature exerts an effect on the oxygen tension necessary to keep up a certain level of metabolism [19]. A higher critical oxygen tension is therefore found at higher temperatures [18]. In this context it is useful to note that limiting oxygen levels in salmonids appear to be high [20, 21]. It is therefore possible that, during growth within the egg capsule with limited oxygen availability, oxygen becomes rate limiting at increased temperatures owing to an increased metabolic rate and oxygen demand. The embryos grown within the egg capsule may thus have suffered from physiological hypoxia.

Energy availability has been shown to affect muscle parameters in mammals. Cheek and Hill [2] reported a reduction in nuclear proliferation and a normal to increased cell size in rats fed on a energy restricted diet with adequate protein supply. In the salmon this response may be advantageous since a similar size can be reached by a certain development stage despite different amounts of energy being available. It is known that the chances of survival of fish larvae are increased if they reach a certain size by the time of first feeding [11].

A larger white muscle fibre cross-sectional area is found for the lower temperature amongst the dechorionated animals. This may be due to a higher level of activity at the higher temperature leading to a higher metabolic intensity [1, 19]. In experiments on the effect of cyanide, increased yolk utilisation efficiency has been linked to reduced activity [14]. For a given rate of yolk absorption a decrease in yolk utilisation efficiency combined with a higher energy demand may have been compensated for by reduced hypertrophic growth. Interestingly, it has been found in Atlantic salmon yolk sac larvae that yolk absorption rates are even reduced in active larvae when compared with inactive larvae [9]. A reduced efficiency due to increased activity may have led to proportionately reduced hypertrophic growth in the high-temperature dechorionated embryos when compared with their lower-temperature counterparts.

The total white muscle cross-sectional area in the presence of the egg capsule was found to be less at 6.5°C than at 11°C. This is in contrast to the results of Stickland et al. [24] and Usher et al. [26] and further emphasises that muscle development in Atlantic salmon is very finely tuned. Stickland et al. [24] used ambient river temperature as the low temperature condition throughout, whereas we used a constant low temperature (6.5°C). Ambient river temperature is variable and it tends to rise towards spring when hatching takes place under natural conditions. The variable temperature regime may improve growth in the low temperature group. It has indeed been found that embryos grown at 6–7°C before hatching and at 8–12°C after hatching showed improved growth when compared with fish reared at a constant temperature of 12°C [17]. Another factor is that flowing water was used by Usher et al. [26] during the incubation. Reduced water velocity reduces embryo size [20] independent of oxygen concentration. This effect seems to have been stronger for the low temperature group than for the high temperature group in this study. Interestingly, however, Usher et al. [26] also found a reduced cross-sectional area at the low temperature at stage 31, although this was not statistically significant.

It is interesting in this context that in chorionated herring *(Clupea harengus)* there is a proportionate increase in muscle fibre hyperplasia with increasing temperature [27]. This may indicate an influence of survival strategies and natural habitat. Herring eggs are substantially smaller than salmon eggs leading to a more favourable surface:volume ratio. Additionally herring eggs are deposited on the surface of rocks, gravel or algae, and are thus exposed to open water whereas salmon eggs are buried in redds. Combined with a short incubation period (10–15 days for herring as opposed to 50–55 days for salmon at 10°C) this may cause oxygen not to become rate limiting in the herring.

Immediate post-hatch growth

The embryos grown within the egg capsule in this study hatched at stage 30. The results found at stage 33 therefore represent the immediate post-hatch growth of the fish.

Whereas salmon raised at 11°C in the chorionated state exhibited a smaller fibre number at stage 31 this difference had disappeared by stage 33. A larger number of fibres was therefore formed between the two stages in this group than in the other groups. As mentioned above, hyperplasia may be linked to energy availability, so that the embryos in the encapsulated state are subject to oxygen, and therefore energy, restriction. With hatching this restraint was removed so that the embryos could then undergo increased fibre hyperplasia. In the lower temperature group and the groups grown without the egg capsule no such effect would have been seen because no comparable oxygen restriction was experienced prior to hatching.

The 11°C chorionated group at stage 33 exhibited relatively less fibre hypertrophy when compared with the other groups. This may be due to the higher rate of hy-

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The influence of temperature on mRNA levels for muscle contractile protein and a proto-oncogene associated with cell division in Atlantic salmon (*Salmo salar* L.)

T.W. Matschak and N.C. Stickland

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The influence of temperature on mRNA levels for muscle contractile protein and a proto-oncogene associated with cell division in Atlantic salmon (*Salmo salar* L.)

T.W. Matschak and N.C. Stickland

Abstract: In Atlantic salmon (*Salmo salar* L.) embryos, an increase in temperature from 5 to 8 or 11°C causes, by the time of hatching, an increase in myofibrillar protein content and a reduction in nuclear proliferation when compared with embryos raised at lower temperatures. This implies a shift in emphasis during embryonic development from nuclear hyperplasia to contractile protein accretion at higher temperatures. The steady-state mRNA levels of proteins indicative of these processes were investigated. The proteins were actin, as a measure for myofibrillar protein, and cellular *myc*, as a measure for nuclear proliferation. Additionally a probe for myosin heavy chain was used. The mRNA levels for myofibrillar protein were not increased at the higher temperature, thus suggesting temperature dependent translational or post translational control. Actin and myosin heavy chain mRNA responses differed, supporting the existence of temperature-dependent myosin heavy chain isoforms. The presence of c-*myc* mRNA in embryonic Atlantic salmon was shown. The mRNA level of this nuclear proliferation marker was decreased at higher temperatures and could be related to histological data on nuclear numbers in embryonic salmon muscle.

Résumé : Chez les embryons de saumon atlantique (*Salmo salar*), une augmentation de la température de 5 à 8 ou 11°C cause, au moment de l'éclosion, une augmentation de la teneur en protéines myofibrillaires et une réduction de la prolifération nucléaire par rapport aux valeurs observées chez des embryons élevés à des températures inférieures. Cela indique qu'au cours du développement de l'embryon, l'accrétion des protéines contractiles devient plus importante que l'hyperplasie nucléaire aux températures plus élevées. On a étudié les teneurs à l'équilibre en mRNA des protéines caractérisant ces processus. Les protéines étaient l'actine, utilisée pour mesurer les protéines myofibrillaires, et le *myc* cellulaire, qui sert à mesurer la prolifération nucléaire. On a également utilisé une sonde sensible à la chaîne à poids moléculaire élevé de la myosine. Les teneurs en mRNA pour les protéines myofibrillaires n'augmentaient pas en fonction de la température, ce qui suggère un mécanisme de régulation dépendant de la température pendant ou après la translation. Les réponses de l'actine et de la chaîne à poids moléculaire élevé de la myosine différaient, ce qui appuie l'hypothèse de l'existence d'isoformes de la chaîne à poids moléculaire élevé de la myosine dépendant de la température. On a démontré la présence de mRNA dans le c-*myc* des embryons de saumon atlantique. La teneur en mRNA de ce marqueur de la prolifération nucléaire diminuait aux températures plus élevées et pourrait être reliée à des données histologiques sur les valeurs nucléaires mesurées dans les tissus musculaires des embryons de saumons.
[Traduit par la Rédaction]

troduction

h are ectothermic animals and embryonic muscle growth, e many other developmental processes such as those con-ned with meristic variation and organ growth, is influenced temperature (Blaxter 1988; Hayes et al. 1953; Johnston 93). Muscle growth and development occur by an increase he size of individual fibres (muscle fibre hypertrophy) and the addition of new muscle fibres (muscle fibre hyper-sia). Both processes necessitate the addition of nuclei to muscle tissue (Cardasis and Cooper 1975; Enesco and Puddy 1964; Nag and Nursall 1972) and, in fish, both play an important role throughout a large proportion of the animals' lives (Stickland 1983; Weatherly and Gill 1985; Vegetti et al. 1990).

In Atlantic salmon (*Salmo salar* L.) temperature exerts a dramatic influence on the muscle cellularity in embryonic stages; temperatures of only 3–6°C above ambient lead to a greater increase in hypertrophic growth relative to muscle fiber hyperplasia (Stickland et al. 1988; Usher et al. 1994). In other words, embryos at a developmental stage around the time of hatching have, in cross section, larger but fewer muscle fibres at the higher temperatures without overall muscle cross-sectional area being affected. Usher et al. (1994) found that this is associated, in Atlantic salmon embryos, with higher myofibrillar protein accretion, as determined by proportional myofibrillar area in white muscle fibers, and a lower number of muscle nuclei per cross-sectional area by the time of hatching at higher temperatures. The mechanism for this change in

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T.W. Matschak[1] and N.C. Stickland. Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, United Kingdom.

[1] Author to whom all correspondence should be addressed. e-mail: tmatscha@rvc.ac.uk

emphasis from nuclear proliferation to myofibrillar protein accretion is unknown.

Fish myosin heavy chain and actin DNA sequences have recently become available (e.g., Gerlach et al. 1990). This represents an opportunity to directly analyze muscle contractile protein mRNA levels whereas previous investigations in fish larvae have mainly focused on total body RNA and protein levels to study growth (e.g., Buckley 1984; Hovenkamp and Witte 1991; Westerman and Holt 1988; Mathers et al. 1993). The effect of temperature on contractile protein mRNA levels combined with the known temperature response of contractile protein accretion make it possible to judge whether regulation takes place pretranslationally, or alternatively affects translation or protein stability. One aim of this study was therefore to gain information on the regulatory steps in the temperature response of contractile protein synthesis in embryonic salmon.

The origin of new muscle nuclei in fish is still unclear. Myoblastic cells (sometimes also referred to as presumptive satellite cells (Johnston 1993)) or satellite cells are generally regarded as their origin (Campion 1984). However, in carp (*Cyprinus carpio* L.) there is a lack of proliferating satellite cells (Koumans et al. 1991, 1994), and amitotic division has been proposed as a mechanism in amphibia (Boujelida and Munk 1987). Early response genes such as cellular *myc* (c-*myc*) are associated with cell division (Cole 1986; Littlewood and Evan 1990). This proto-oncogene has been found in rainbow trout (*Oncorhynchus mykiss* Walbaum), a salmonid, and its sequence is available (van Beneden et al. 1986). A further aim of this study was therefore to assess the presence of c-*myc* in Atlantic salmon and to investigate how it relates to the nuclear proliferation patterns found at different temperatures.

Materials and methods

Animals

The Atlantic salmon embryos were derived from a single adult pairing from the same stock as the fish used by Usher et al. (1994). After fertilization the eggs were divided into three groups and kept either at ambient river temperature or in thermostat-regulated water at 8 or 11°C at the SOAFD Freshwater Fisheries Laboratory, Almondbank, Perthshire, Scotland. The ambient river temperature corresponded to an average daily temperature of 3.4°C.

At Gorodilov (1983) stages 25, 27, 29, 31, and 33 the embryos were sent to London by rail in water of the appropriate temperature insulated in polystyrene containers. The eggs were then kept in incubators at 5°C for the ambient river temperature group or at 8 and 11°C, as appropriate, for at least 24 h to let the embryos recover from any fluctuation in temperature during transport.

At the appropriate sampling time the salmon eggs were dechorionated if necessary, the embryos were killed by decapitation, and the yolk sac was removed so that the remaining tissue consisted mainly of muscle. The tissue was stored in liquid nitrogen until further use. Because of the small size of the fish, 10 to 15 embryos were pooled per sample.

RNA isolation and fractionation

The RNA was isolated as described by Chomczynski and Sacchi (1987). The amount of RNA present was estimated by determining the optical density of the solution at 260 nm (OD_{260}) and purity was estimated from the ratio OD_{260}/OD_{280}. The RNA was then immediately fractionated on a denaturing agarose gel and blotted according to the modified method of Fourney et al. (1988). Briefly, 20 µg of total RNA were made up to a volume of 15 µL with deionized H_2O treated with diethyl pyrocarbonate and then 15 µL of loading buffer without bromophenol blue was added. Ethidium bromide was added to a 1% agarose gel at a final concentration of 0.01%. The gel was run at 4°C for 20 h with a voltage of 30 V and then photographed on a UV transilluminator. Finally, the RNA was blotted onto nylon membrane (Hybond N, Amersham) using standard methods (Sambrook et al. 1989). The RNA was fixed to the membrane by baking at 80°C for 2 h.

DNA probes

For muscle contractile protein a carp actin probe, specific for both alpha and beta actins (FGA101, Gerlach et al. 1990), and a carp myosin heavy chain (MyHC) probe, corresponding to exon 40 (FG2Ex40), were used and labelled with ^{32}P by random priming (Amersham Multiprime DNA labelling system). The actin probe had been shown to correspond to a highly conserved region in its respective gene (Gerlach et al. 1990). The MyHC probe corresponded to exon 40 in the relatively conserved rod region.

An oligonucleotide (48-mer) corresponding to a conserved region, nucleotide 778 to nucleotide 825, of the rainbow trout cellular c-*myc* gene (van Beneden et al. 1986) served as a probe for c-*myc*. The probe was 3′-end labelled with ^{32}P as described (Titus 1991) using terminal transferase (enzyme and buffer from Pharmacia).

Northern hybridization

The Northern hybridization was carried out as recommended by the membrane manufacturer (Amersham). Briefly, the hybridization took place at 42°C in 5× SSPE buffer containing 5× Denhardt's solution and 0.5% sodium lauryl sulphate (SDS) at a final concentration of 50% formamide. Washes were carried out under low stringency conditions, i.e., at 65°C and with 1× SSPE, 0.1% SDS buffer, with the exception of the hybridization reaction with the c-*myc* probe, which was washed at 55°C. After autoradiography the membrane was stripped of bound probe twice by pouring on boiling SDS solution (0.1%) and then reused for further experiments.

Evaluation

The washed blots were analyzed by autoradiography at –70°C employing intensifying screens (Amersham). To be able to quantify the results the films were preflashed to give a linear response to the amount of radioactivity present.

The autoradiographs were analyzed using a Seescan image analyzer to determine the integral optical density for each sample and probe. The results were normalized against the amounts of ribosomal RNA (rRNA) present, as determined from the amount of ethidium bromide bound to the nucleic acid, to compensate for possible pipetting errors. Ribosomal RNA represents the bulk of RNA present in total RNA (Young 1976) and therefore is a good measure for the amount of the latter. It is routinely used as an internal control in Northern blotting (see for example Hesketh et al. 1992; Iwaki et al. 1990). For the measurements of rRNA a negative image of the UV-transilluminated agarose gel was produced from a photograph and analyzed in the same way as the autoradiographs. The values for both species (28S and 18S) of rRNA were combined. The size of the RNA species identified was estimated by comparison with the migration distances of the 28S and 18S rRNA.

Statistics

The data were analyzed by two-way analysis of variance and the means were compared by the Newman–Keuls method (Snedecor and Cochran 1969).

Results

Northern hybridization

Figure 1 shows the autoradiographs for the various probes

used and the agarose gel stained with ethidium bromide. As can be seen, clear specific bands of the appropriate sizes were detected. There was almost no variation in the amount of total RNA pipetted onto the gel (Fig. 1D).

The cDNA specific for carp muscle contractile protein transcript hybridized to the salmon RNA. The probes bound only to bands of the expected size and no nonspecific binding was observed. With progressing development the relative amount of specific messenger RNA (mRNA) was found to increase. This was more pronounced for actin ($p < 0.001$) than for MyHC ($p = 0.029$) (Figs. 2A and 2B). At stage 31 (around the time of hatching) no such increase of contractile protein mRNA with increasing developmental age was observed at ambient temperature and 8°C. For both actin and MyHC a statistically highly significant ($p < 0.001$) decrease in mRNA steady state levels was observed at 8 and 11°C compared with at ambient temperature. However, no statistically significant difference in contractile protein mRNA levels was found between these two elevated temperatures. A comparison between the mRNA levels for MyHC and actin shows that there was proportionately more signal for MyHC than for the actin probe the lower the temperature ($p < 0.001$), whereas there was relatively little to no change with development ($p = 0.103$) (Fig. 2C).

The c-*myc* transcript was clearly present in the embryos with lower temperature fish exhibiting a higher steady state level ($p = 0.002$) (Fig. 2D). Embryos raised at 11 and 8°C behaved in a very similar way, with the 8°C fish showing only a marginally stronger signal at stage 33 (not significant). At ambient temperature an increase with development appeared to be present whereas both 8 and 11°C mRNA levels remained relatively constant.

The proportionate increase of actin mRNA in comparison with c-*myc* mRNA was calculated as a ratio. The result is shown in Fig. 2E. More c-*myc* relative to actin mRNA was present at the ambient temperature ($p < 0.05$). No statistically significant change with development was found ($p = 0.789$).

Discussion

Both carp probes (actin and MyHC) were found to cross-react with the appropriate salmon RNA species. In the case of the actin probe, cross-reaction was not surprising because only two striated muscle actin isoforms are known (Buckingham 1985) and the actin probe used has been shown to correspond to a highly conserved region within the molecule (Gerlach et al. 1990). In Fig. 2B two actin bands appear to be present. The size difference between these bands is smaller than would be expected between α-actin and cytoskeletal actin, and also smaller than the difference found for α- and β-actin in carp by Gerlach et al. (1990). No heart tissue was present in the samples and the two bands were thus combined and assumed to represent myofibrillar actin. The data obtained for actin should therefore represent a good measure for muscle contractile protein mRNA. As has been found in mammals (Buckingham 1985) the mRNA levels increased with development at all three temperature regimes used. Interestingly, the amount of contractile protein mRNA remained at the same level for 8°C and ambient river temperature at stage 31 when compared with stage 29. In the salmon embryos used, stage 31 coincided with the hatching period. Hatching has been associated with a reduction in growth and this may be responsible for this particular finding (Rombough 1988).

Embryos reared at a temperature above the ambient river temperature did not exhibit higher contractile protein mRNA levels; on the contrary, a lower level of steady state mRNA for actin or MyHC was found at the higher temperatures for all developmental stages investigated. Usher et al. (1994) found higher protein accretion at an increased temperature (11°C against 5 or 8°C) for the same stock of fish and it would thus seem that this higher myofibrillar protein content is effected

Fig. 1. Autoradiographs of a nylon membrane probed with DNA specific for (A) myosin heavy chain (exon 40) and (B) actin under low stringency conditions (1× SSC, 0.1% SDS, 65°C), and (C) c-*myc* (1× SSC, 0.1% SDS, 55°C; note the c-*myc* specific bands near the position of the 18S rRNA). In (D) the level of ethidium bromide bound to the ribosomal RNA in the gel used for the above blots is shown. Twenty micrograms of total RNA was applied to each lane. The positions of the 28S and 18S ribosomal mRNAs are shown. From left to right, developmental stages 25, 27, 29, 31, and 33 were applied for each temperature.

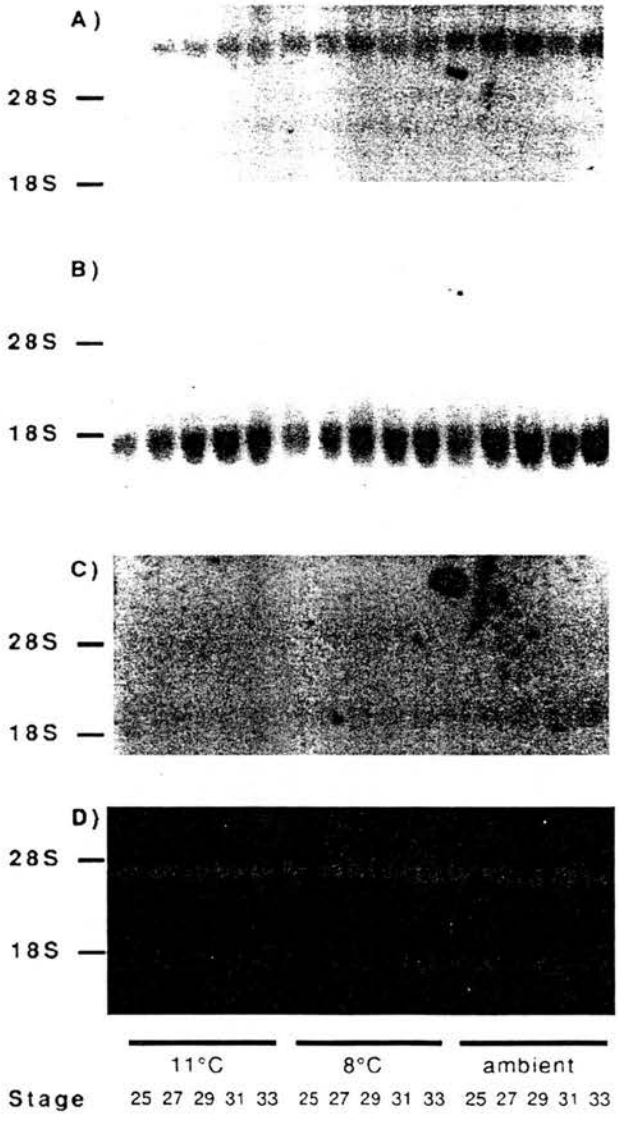
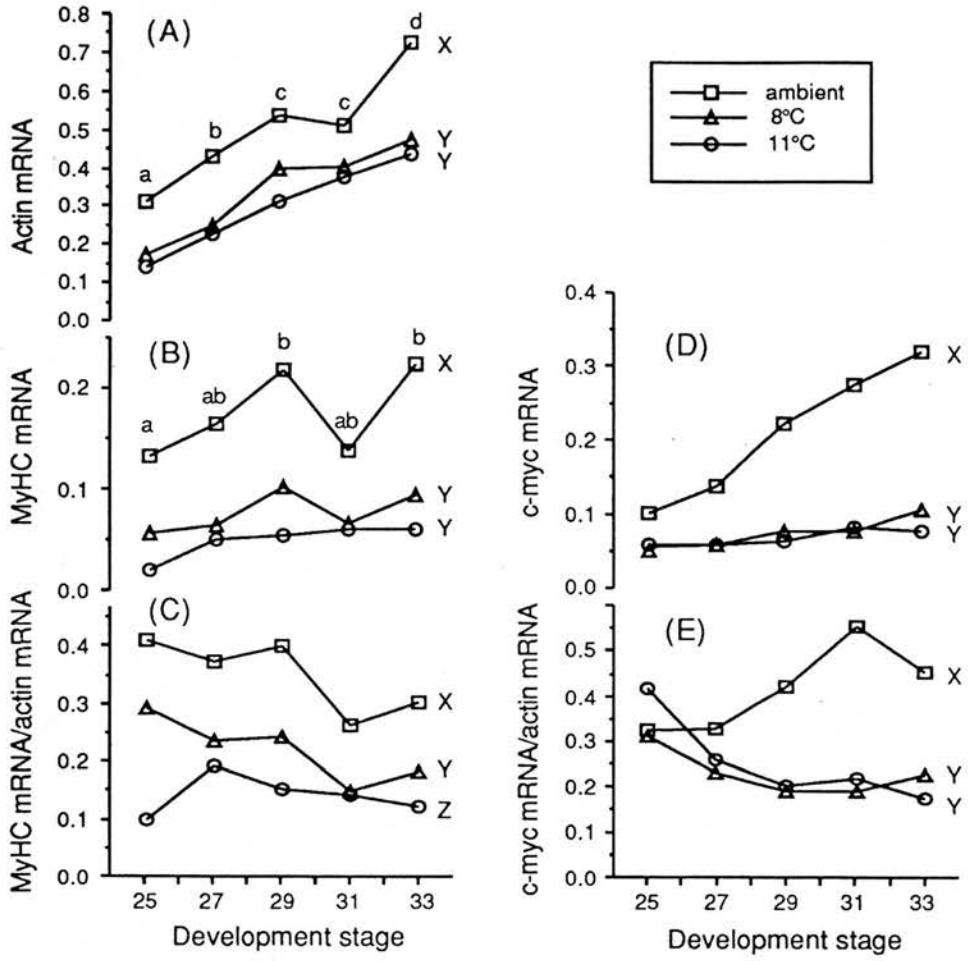


Fig. 2. (A) Actin, (B) myosin heavy chain (MyHC), and (D) c-*myc* steady state mRNA levels for the five different developmental stages and the three temperatures employed. The amounts are expressed as relative optical density on autoradiographs normalized for the amount of ribosomal RNA present. In C and E, the amounts of MyHC mRNA (from B) and c-*myc* (from D), respectively, are shown relative to actin mRNA (from A). A statistically significant difference ($p < 0.05$) is indicated by differing letters (for temperature, uppercase letters; for development, lowercase letters). Ambient temperature averaged 3.4°C over the entire experimental period.



by translational or post-translational control rather than through upregulation of transcription or higher mRNA stability. Evidence for translational control has been found, for example, in the stimulation of hypertrophy in rat gastrocnemius muscle by clenbuterol (Hesketh et al. 1992), or in muscle protein synthesis in cod in response to different ration sizes (von der Decken and Lied 1989). A variation in protein stability, i.e., post-translational control, is, however, also a possible explanation for the differences in protein accretion observed in embryonic salmon. No statistically significant difference in the contractile protein mRNA levels between 8 and 11°C was found in this study, whereas the myofibrillar contents had been found to be different (Usher et al. 1994). This again suggests that the control of contractile protein accretion does not take place before translation.

The MyHC transcript, which was investigated as a second contractile protein mRNA, exhibited a result similar to that found for actin. The embryos grown at ambient river temperature showed the highest level of MyHC mRNA compared with those grown at 11 or 8°C. When the results are directly compared with the results for actin, a higher MyHC/actin ratio is obtained the lower the experimental temperature. Bearing in mind that the number of MyHC isoforms is substantially larger than for actin, this result may be interpreted in the following way. In mammals various developmental isoforms are present (Buckingham 1985) and in carp a minimum of 28 MyHC genes seem to be present and isoform switching may be involved in temperature acclimation (Gerlach et al. 1990). The MyHC probe used corresponds to a region in exon 40 of the gene, which is part of the relatively (but not fully) conserved rod region. The present result may arise because the probe did not, at least in the salmon, hybridize to all isoforms, which would have resulted in a similar MyHC/actin ratio regardless of the temperature treatment. Perhaps, for this reason, the embryonic salmon, like the carp, exhibits an isoform switch at different temperatures. Differences in MyHC isoform depending on water temperature have been reported in a number of studies (e.g., cod: Gerlach et al. 1990; Hwang et al. 1990; Arctic

char: Martinez and Patterson 1992). Alternatively, different translation rates or protein stabilities for actin and MyHC could lead to the observed result. The mRNA data found would then simply represent different transcription rates or mRNA stabilities for actin and total MyHC. As is discussed by Lowery and Somero (1990) for starvation in *Paralabrax nebulifer*, complex changes in ribosomal activity and capacity combined with changes in protein turnover rates can differentially affect protein levels. Unfortunately no salmon probes for the 3′ untranslated region of the MyHC gene, which would make it possible to clarify the situation, are available as yet.

The expression of c-*myc* has been found to be associated with the entry of cells into the cell cycle and correlates with the rate of cell proliferation (reviewed in Cole 1986 and Littlewood and Evan 1990). Furthermore, the amount of c-*myc* protein present directly correlates with mRNA levels (Hann and Eisenmann 1984) and its protein half-life does not change during growth (Persson et al. 1985). In muscle, c-*myc* expression declines with the onset of differentiation and plays a role in the control of proliferation and differentiation (reviewed in Olson 1992). Both c-*myc* mRNA and protein have a very short half-life and have to be expressed throughout the cell cycle (Littlewood and Evan 1990). Additionally c-*myc* mRNA levels have been shown to fall with the replacement of hyperplasia with hypertrophy in mouse forebrain, hindbrain, and kidney (Zimmerman et al. 1986). The c-*myc* transcript was clearly present and more prevalent at the lower temperature, indicating a higher level of nuclear proliferation in this case. This reflects the result found for nuclear numbers at hatching for different temperatures in the same stock of fish (Usher et al. 1994).

A comparison of the steady state mRNA levels for actin as a measure for contractile muscle protein and c-*myc* as a measure for nuclear proliferation showed a higher c-*myc*/actin ratio at ambient temperature than at 11°C. This difference appears to be present only from stage 29 onwards, but no statistical difference between the developmental stages was found. The result would be consistent with the histological findings of Usher et al. (1994) who found no difference in nuclear numbers and myofibrillar area at stage 25 but a significant difference at stage 33. However, it should be emphasized that the actin mRNA levels do not relate directly to the contractile protein levels.

In summary, this study has demonstrated a reduction in contractile protein mRNA (actin and MyHC) at increased temperatures and suggests that contractile protein accretion in embryonic Atlantic salmon is achieved by translational or post-translational control rather than at the transcriptional or mRNA stability level. The presence of c-*myc* mRNA was demonstrated and the results show a relative reduction in its level with an increase in temperature.

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References

Blaxter, J.H.S. 1988. Pattern and variety in development. *Edited by* W.S. Hoar and D.J. Randall. Fish physiology. Vol. XI. Part A. Academic Press, London. pp. 1–58.

Boujelida, H., and Munk, L. 1987. Multinucleation during myogenesis of the myotome of *Xenopus laevis*: a qualitative study. Development (Cambridge), **101**: 583–590.

Buckingham, M. 1985. Actin and myosin multigene families: their expression during the formation of skeletal muscle. Essays Biochem. **20**: 77–109.

Buckley, L.J. 1984. RNA–DNA ratio: an index of larval fish growth in the sea. Mar. Biol. (Berlin), **80**: 291–298.

Campion, D.R. 1984. The muscle satellite cell: a review. Int. Rev. Cytol. **87**: 225–251.

Cardasis, C.E., and Cooper, G.W. 1975. An analysis of nuclear numbers on individual muscle fibres during differentiation and growth: a satellite cell – muscle fibre growth unit. J. Exp. Zool. **191**: 347–358.

Chomczynski, P., and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**: 156–159.

Cole, M.D. 1986. The myc oncogene: its role in transformation and differentiation. Annu. Rev. Genet. **20**: 361–384.

Enesco, M., and Puddy, D. 1964. Increase in the number of nuclei and weight in skeletal muscle of rats of various ages. Am. J. Anat. **114**: 235–244.

Fourney, R.M., Miyakoshi, J., Day, R.S., III, and Paterson, M.C. 1988. Northern blotting: efficient RNA staining and transfer. Betheseda Res. Lab. Focus, **10**: 5–6.

Gerlach, G.-F., Turay, L., Malik, K.T.A., Lida, J., Scutt, A., and Goldspink, G. 1990. Mechanisms of temperature acclimation in the carp; a molecular biology approach. Am. J. Physiol. **259**: R237–R244.

Gorodilov, Yu.N. 1983. Stadii embryonalnogo razvitiya atlanticheskogo lososya *Salmo salar* L. II. Opisaniye i khronologia. Sb. Nauchn. Tr. Gosniorkh. **200**: 107–126. (Translated by J.E. Thorpe. Scott. Fish. Res. Transl. **19**: 1–34.)

Hann, S.R., and Eisenmann, R.N. 1984. Proteins encoded by the human c-*myc* oncogene: differential expression in neoplastic cells. Mol. Cell. Biol. **4**: 2486–2497.

Hayes, F.R., Pelluet, D., and Gorham, E. 1953. Some effects of temperature on the embryonic development of the salmon (*Salmo salar*). Can. J. Zool. **31**: 41–52.

Hesketh, J.E., Campbell, G.P., Lobley, G.E., Maltin, C.A., Acamovic, F., and Palmer, R.M. 1992. Stimulation of actin and myosin synthesis in rat gastrocnemius muscle by clenbuterol; evidence for translational control. Comp. Biochem. Physiol. C, **102**: 23–27.

Hovenkamp, F., and Witte, J.I.J. 1991. Growth, otolith growth and RNA/DNA ratios of larval plaice, *Pleuronectes platessa*, in the North Sea, 1987 to 1988. Mar. Ecol. Prog. Ser. **70**: 105–116.

Hwang, G.C., Watabe, S., and Hashimoto, K. 1990. Changes in carp myosin ATPase induced by temperature acclimation. J. Comp. Physiol. B, **160**: 233–239.

Iwaki, K., Sukhatme, V.P., Shubeita, H.E., and Chien, K.R. 1990. α- and β-adrenergic stimulation includes distinct patterns of immediate early gene expression in neonatal rat myocardial cells. J. Biol. Chem. **265**: 13 809 – 13 817.

Johnston, I.A. 1993. Temperature influences muscle differentiation and the relative timing of organogenesis in herring (*Clupea harengus*) larvae. Mar. Biol. (Berlin), **116**: 363–379.

Koumans, J.T.M., Akster, H.A., Booms, R.G.H., Lemmens, C.J.J., and Osse, J.W.M. 1991. Numbers of myosatellite cells in white

axial muscle of growing fish: *Cyprinus carpio* L. (Teleostei). Am. J. Anat. **192**: 418–424.

Koumans, J.T.M., Akster, H.A., Booms, G.H.R., and Osse, J.W.M. 1994. Numbers of muscle nuclei and myosatellite cell nuclei in red and white axial muscle during growth of the carp (*Cyprinus carpio* L.). J. Fish Biol. **44**: 391–408.

Littlewood, T.D., and Evan, G.I. 1990. The role of *myc* oncogenes in cell growth and differentiation. Adv. Dent. Res. **4**: 69–79.

Lowery, M.S., and Somero, G.N. 1990. Starvation effects on protein synthesis in red and white muscle of the barred sand bass, *Pleuronectes nebulifer*. Physiol. Zool. **63**: 630–648.

Martinez, I., and Petterson, G.W. 1992 Temperature-induced precocious transitions of myosin heavy chain isoforms on the white muscle of the Arctic charr *Salvelinus alpinus* (L.). Basic Appl. Myol. **2**: 89–95.

Mathers, E.M., Houlihan, D.F., McCarthy, I.D., and Burren, L.J. 1993. Rates of growth and protein synthesis correlated with nucleic acid content in fry of rainbow trout, *Oncorhynchus mykiss*; effects of age and temperature. J. Fish Biol. **43**: 245–263.

Nag, A.C., and Nursall, J.R. 1972. Histogenesis of white and red muscle fibers of trunk muscles of a fish *Salmo gairdneri*. Cytobios, **6**: 227–246.

Olson, E.N. 1992. Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. **154** : 261–272.

Persson, H., Gray, H.E., and Godeau, F. 1985. Growth dependent synthesis of c-*myc*-encoded proteins: early stimulation by serum factors in synchronised mouse 3T3 cells. Mol. Cell. Biol. **5**: 2903–2912.

Plombough, P.J. 1988. Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. *In* Fish physiology. Vol. XI. Part A. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, London. pp. 59–161.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. pp. 7.46–7.50.

Snedecor, G.W., and Cochran, W.G. 1969. Statistical methods. 6th ed. The Iowa State University Press, Ames, Iowa. pp. 299–338.

Stickland, N.C. 1983. Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*). J. Anat. **137**: 323–333.

Stickland, N.C., White, R.N., Mescall, P.E., Crook, A.R., and Thorpe, J.E. 1988. The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L.). Anat. Embryol. **178**: 253–257.

Titus, D.E. 1991. Promega protocols and applications guide. Promega Corporation, Madison, Wis. p. 148.

Usher, M.L., Stickland, N.C., and Thorpe, J.E. 1994. Muscle development in Atlantic salmon (*Salmo salar*) embryos and the effect of temperature on muscle cellularity. J. Fish Biol. **44**: 953–964.

van Beneden, R.J., Watson, D.K., Chen, T.T., Lautenberger, J.A., and Papas, T.S. 1986. Cellular *myc* (c-*myc*) in fish (rainbow trout): its relationship to other vertebrate *myc* genes and to the transforming genes of the MC29 family of viruses. Proc. Natl. Acad. Sci. U.S.A. **83**: 3698–3702.

Vegetti, A., Mascarello, F., Scapolo, P.A., and Rowlerson, A. 1990. Hyperplastic and hypertrophic growth of lateral muscle in *Dicentrarchus labrax* (L.). An ultrastructural and morphometric study. Anat. Embryol. **182**: 1–10.

von der Decken, A., and Lied, E. 1989. Myosin heavy chain synthesis in white trunk muscle of cod (*Gadus morhua*) fed different ration sizes. Fish Physiol. Biochem. **6**: 333–340.

Weatherly, A.H., and Gill, H.S. 1985. Dynamics of increase in muscle fibers in fishes in relation to size and growth. Experientia, **41**: 353–354.

Westerman, M.E., and Holt, G.J. 1988. The RNA–DNA ratio: measurement of nucleic acids in larval *Sciaenops ocellatus*. Contrib. Mar. Sci. **30**(Suppl.): 479–485.

Young, V.R. 1976. The role of skeletal and cardiac muscle in the regulation of protein metabolism. *In* Mammalian protein metabolism. Vol. 4. *Edited by* H.N. Munro. Academic Press, London. pp. 586–674.

Zimmerman, K.A., Yancopoulos, G.D., Collum, R.G., Smith, R.K., Kohl, N.E., Denis, K.A., Nau, M.M., White, O.N., Toran-Allerand, D., Gee, C.E., Minna, J.D., and Alt, F.W. 1986. Differential expression of *myc* family genes during murine development. Nature (London), **319**: 780–783.

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Activity of cytochrome *c* oxidase and lactate dehydrogenase in muscle tissue of slow growing (lower modal group) and fast growing (upper modal group) Atlantic salmon

C. NATHANAILIDES* AND N. C. STICKLAND

Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London, Royal College Street, London NW1 0TU, U.K.

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Fast growing Atlantic salmon (upper modal group) exhibit a higher activity of muscle cytochrome *c* oxidase (CCO) and lactate dehydrogenase (LDH) than the slow growing salmon (lower modal group). The ratios of CCO/LDH activity, indicate a higher aerobic/anaerobic metabolic potential of the axial muscle in the upper modal group.

Key words: bioenergetics; aerobic metabolism; bimodality.

A growth bimodality occurs in salmon juveniles *Salmo salar* L., during the first winter of their life: some fish cease feeding and growing and become the lower modal group fish (LMG), whereas fish of the upper modal group (UMG) continue to feed and grow (Thorpe, 1977; Thorpe *et al.*, 1982; Metcalfe *et al.*, 1986). Cytochrome *c* oxidase (CCO) is a mitochondrial enzyme which exhibits higher activity in fast growing fish (Houlihan *et al.*, 1993) and its activity can be used as an indicator of the aerobic metabolism of fish muscle (Goolish & Adelman, 1987). Usually in studies of this field, different growth rates have been induced experimentally by altering the feeding regime or temperature. In this study, all fish were reared under identical conditions. The specific aim of the present study was to test the hypothesis that salmon muscle tissue of the LMG exhibits a lower capacity for aerobic metabolism than the UMG fish.

Samples of 0+ Atlantic salmon of the LMG and UMG were obtained in March 1993 from the local stock (and of the same age) held in the Freshwater Fisheries Laboratory, Almondbank, Pitlochry, Scotland. Five fish were sampled from each modal group. The fish of the LMG were significantly smaller than the UMG fish (Table I). White muscle homogenates from five fish of each modal group were prepared and the activity of CCO was assayed according to Tyler & Nathanailides (1995) at 20° C in a medium containing 0·075 M potassium phosphate buffer, pH 6·8 and 0·025 mM ferrocytochrome *c*. Activity of the glycolytic enzyme lactate dehydrogenase (LDH) was assayed in a medium of 50 mM potassium phosphate buffer (pH 7·0), containing 0·15 mM NADH and 0·60 mM sodium pyruvate (omitted from the control). Activity of enzymes is given in units per mg protein. Protein content was estimated using the Folin–Lowry method (Lowry *et al.*, 1951). Results from the LMG and UMG fish were compared by Student's *t*-test; the ratios of CCO/LDH activity were arc–sin transformed prior to statistical analysis.

Activities of the aerobic metabolism enzyme CCO and the glycolytic LDH of the axial muscle were significantly higher in the fast growing UM group. The ratio of CCO/LDH activity of axial muscle was also significantly higher in the UMG salmon (Table I). There is some evidence to suggest that the activity of CCO correlates with aerobic metabolism (Goolish & Adelman, 1987) and that the activity of LDH can correlate with anaerobic metabolic potential (Hochachka *et al.*, 1983). It is therefore possible, that changes in the ratio of mitochondrial enzyme activity/LDH can indicate alterations in the metabolic

*Present address: Sherrington School of Physiology, St. Thomas's Hospital Medical School (UMDS), Lambeth Palace Road, London SE1 7EH, UK.

TABLE I. Body weight (g) and activity of enzymes in the lower (LMG) and upper (UMG) modal group salmon

	UMG	LMG
Body weight	14·54 (1·02)	2·39 (0·19)*
CCO	5·86 (0·45)	2·04 (0·18)*
LDH	27·78 (1·65)	20·60 (1·34)*
CCO/LDH	0·21 (0·02)	$^{-4}$0·09 (4×10^{-4})*

Enzyme activity for CCO is given in μmoles of ferrocytochrome *c* oxidized min^{-1} $mg\ protein^{-1}$, and for LDH as reduction of NADH μmoles min^{-1} $mg\ protein^{-1}$. Differences assessed by Student's *t*-test (*$P<0·001$). Numbers in parentheses indicate the S.E.M., $n=5$. CCO, cytochrome *c* oxidase; LDH, lactate dehydrogenase.

potentials of aerobic and anaerobic metabolism (Hochachka *et al.*, 1983). In the present study, CCO/LDH, was significantly higher in the UMG fish indicating that there was a relatively higher capacity for maximal aerobic metabolism in the UMG, compared with the LMG. A higher maximal capacity for anaerobic glycolysis and aerobic metabolism in the UMG fish, may be the result of feeding-related, higher swimming activity of the UMG fish (Metcalfe *et al.*, 1986). Activity of LDH may increase with size in fish (Somero & Childress, 1980). It is therefore possible that the higher LDH activity of the UMG fish, is simply a result of the size difference between the two modal groups.

In agreement with previous studies (Goolish & Adelman, 1987; Houlihan *et al.*, 1993), the fast growing fish (in the present study the UMG salmon) exhibited a higher rate of muscle CCO activity. In larval stages of salmon, some fish exhibit a higher feeding activity and accelerated rate of growth and development and are likely to be more dominant and become the upper modal group (Metcalfe & Thorpe, 1992). In a recent study (Metcalfe *et al.*, 1995) it was found that the fish which exhibit fast growth and development, also exhibit a higher standard metabolic rate, as measured by oxygen consumption. In support of this finding, the present study showed that the UMG of salmon exhibited increased CCO activity, indicating that the fast growth and development of the UMG is associated with a higher capacity for aerobic metabolism.

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References

Goolish, E. M. & Adelman, I. R. (1987). Tissue specific cytochrome *c* oxidase activity in largemouth bass: the metabolic cost of feeding and growth. *Physiological Zoology* **60**, 454–464.

Higgins, P. J. & Thorpe, J. E. (1990). Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon, (*Salmo salar* L.). *Journal of Fish Biology* **37**, 505–519.

Hochachka, P. W., Stanley, C., Merkt, J. & Sumar-Kalinowski, J. (1983). Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. *Respiratory Physiology* **52**, 303–313.

Houlihan, D. F., Mathers, E. M. & Foster, A. (1993). Biochemical correlates of growth rate in fish. In *Fish Ecophysiology* (Rankin, J. C. R. & Jensen, F. B., eds), pp. 45–67. London: Chapman & Hall.

Lowry, O. H., Rosebrough, N. T., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

Metcalfe, N. B., Huntingford, F. A. & Thorpe, J. E. (1986). Seasonal changes in feeding motivation of juvenile Atlantic salmon (*Salmo salar*). *Canadian Journal of Zoology* **64**, 2439–2446.

Metcalfe, N. B. & Thorpe, J. E. (1992). Early predictors of life-history events: the link between first feeding date, dominance and seaward migration in Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology* **41** (suppl. B), 93–99.

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Muscle cellularity in relation to somatic growth in the European sea bass *Dicentrarchus labrax* (L.)

C Nathanailides[1], O Lopez-Albors[2], E Abellan[3], J M Vazquez[2], D D Tyler[1], A Rowlerson[4] & N C Stickland[1]

[1]Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London, Royal College Street, London NW1 0TU, UK, [2] Dept Anatomy & Embryology, University of Murcia, 30071, Murcia, Spain, [3]Instituto Espanol de Oceanographica (IEO), Planta de Cultivor Marinos, Puerto de Mazarron, Spain, and [4]Department of Physiology, United Medical and Dental Schools, St Thomas's Hospital Campus, University of London, Lambeth Palace Road, London SE1 7EH, UK

Correspondence: Dr C Nathanailides, Fish Nutrition Unit, c/o Dr M. Alexis, National Centre for Marine Research (ΕΚΘΕ), Hellinikon, Athens, TK 16040 Greece.

Abstract

Sea bass, *Dicentrarchus labrax* (L.), juveniles (1.32 g) grew at a rate of 4.2% per day, when fish were fed *ad libitum* and reared at optimal thermal conditions (ambient sea water temperature, 20–26°C). At 13°C, feeding and spontaneous activity were severely restricted and somatic growth was reduced to 0.6% per day. Over a period of 6 weeks, both muscle fibre hyperplasia and nuclear division were higher in the ambient-temperature group compared with the fish reared at 13°C. Despite the differences in growth rate and spontaneous activity, muscle fibre hyperplasia was paralleled by nuclear division in the lateral axial muscle in both temperature groups and the number of nuclei per myofibre did not differ significantly between the two temperature groups. It is concluded that at optimal thermal and feeding conditions, somatic growth of sea bass juveniles is mediated through an increase in the number of nuclei and muscle fibres.

Introduction

Temperature can influence the growth and appetite of fish (Barnabé 1990). For the purpose of aquaculture, fish muscle growth is important. In juvenile stages, fast fish growth is associated with recruitment of new myofibres (hyperplasia) (Stickland 1983; Weatherley & Gill 1981), which may originate from myosatellite cells or other myogenic cells (Koumans & Akster 1995). Muscle fibre hyperplasia can be influenced by feeding regimes (Kiessling, Storebakken, Asgard and Kiessling 1991). It is believed that after a critical ratio of nuclei to cytoplasm is reached, muscle fibre hypertrophy also requires the addition of new myonuclei (Cardasis & Cooper 1975) derived from muscle myoblasts. Thus proliferation of myogenic cells supports two distinct processes that may respond differently to thermal conditions. For example, temperature can influence the rate of muscle fibre hyperplasia (Weatherley & Gill 1979) and the rate of *in vitro* differentiation of myosatellite cells (Matschack & Stickland 1995). In the early life stages of a stenothermal fish species, the Atlantic salmon *Salmo salar* L., nuclear division and muscle fibre hyperplasia are inversely related to rearing temperature (Nathanailides, Lopez-Albors and Stickland 1995a).

Sea bass *Dicentrarchus labrax* (L.) is a eurythermic species, widely distributed from the Atlantic coast of Morocco to the North Sea (Picket & Pawson 1994) and it is extensively cultivated in the Mediterranean sea. When fed *ad libitum*, the daily growth rate of sea bass can range from under 0.4% to more than 5% depending on the temperature (Barnabe 1990). Sea bass exhibits a rapid growth during post larval stages, with both muscle hyperplasia and hypertrophy contributing to muscle growth (Veggetti, Mascarello, Scapolo & Rowlerson

1990). Temperature is a controlling factor (Fry 1971) of spontaneous swimming activity, metabolic rate, feeding and growth. In the present study, the muscle growth and cellularity of sea bass reared at optimal and suboptimal thermal conditions was investigated.

Materials and methods

Rearing of fish

Sea bass juveniles (1.32 ± 0.47 g) originating from the Spanish Oceanographic Institute (IEO) of Murcia (Puerto de Mazarron) breeding station, and reared at ambient sea water temperature (fluctuating between 16 and 20°C) were used in the present study. A sample of 120 fish was selected randomly.

An initial sample of fish was obtained as a control group (week 0) and the remaining fish were divided into two groups. One group of about 50 fish was reared at the ambient sea water temperature of the IEO for 6 weeks, which increased from 20°C in week 0 to 26°C in week 6 (warm temperature group), and another group of about 50 fish was reared in sea water cooled to 13°C (±0.7) for 6 weeks (cold temperature group). Fish were fed *ad libitum* twice a day with commercially obtained sea bass feed. Samples of fish were examined at the start of the acclimation period (week 0, i.e. the control group) and at 4 and 6 weeks later, from each temperature group.

The daily specific growth rate (SGR%) was estimated according to the following equation (ln BW_2 − ln BW_1) × days^{-1}. BW_2 and BW_1 are body weights at week 6 and week 4 respectively.

Muscle cellularity

Muscle tissue samples (n = 5–7 fish per group) of whole transverse sections of the post-anal caudal region (about 3–5 mm thick) were obtained at weeks 0, 4 and 6. The samples were fixed in a solution of 10% phosphate-buffered formol saline, dehydrated in a graded series of ethanol solutions and embedded in glycol methacrylate. Whole transverse sections (7 µm thick) were obtained and stained with Weigert-Van Gieson stain. Proliferation of myofibres in sea bass juveniles occurs throughout the lateral muscle (Veggetti *et al.* 1990). In the present study, the cross-sectional area (CSA) of individual myofibres (n > 150 per fish for each stage in each group) from the deep white muscle in the epaxial region was traced using a computerised image analysis system. The number of nuclei was counted manually on the screen, and the number of nuclei per frame CSA (ranging from 1.6×10^5 to 2.8×10^5 µm^2, depending on fish size) was extrapolated to produce total values for the whole CSA of axial muscle per fish. The total number of myofibres contained in the whole cross-section of each fish was estimated, according to Higgins & Thorpe (1990), by using the ratio of whole muscle CSA to mean myofibre size. Statistical analysis (ANOVA) was used to determine the existence of significant differences ($P < 0.05$) between week 0 and weeks 4 or 6 in the measured parameters. Student's t-test was used for testing the significance of differences in the measured parameters between the two temperature groups at weeks 4 and 6.

Results

Somatic growth

The fish reared at ambient temperature grew faster (SGR = 4.2%) than the fish reared at 13°C (SGR = 0.6%). As a result, after 6 weeks the fish in the ambient temperature group exhibited a mean body weight of 7.5 ± 0.5 g whereas the fish reared at 13°C exhibited a mean body weight of 1.7 ± 0.1 g.

Muscle cellularity

After 4 and 6 weeks, the mean myofibre cross sectional area (MCSA) of white muscle was not significantly different between the two groups (Fig. 1a), but in the 13°C group there was a reduction in the mean myofibre size between week 0 and week 6 (ANOVA, $P = 0.032$). The total number of myofibres per fish CSA increased in both groups during the 6 weeks period. By the fourth week, the fish reared at ambient temperature exhibited a significantly increased muscle fibre hyperplasia, as shown by the total number of myofibres, compared with the fish reared at 13°C (Fig. 1b).

The total CSA per fish increased by a factor of 3.5 in the ambient temperature group and by a factor of 1.2 in the 13°C group (Fig. 1c and Table 1). The number of nuclei per whole CSA of the axial muscle of fish reared at ambient temperature was significantly higher than that in fish reared at 13°C (Fig. 1d). The number of nuclei per myofibre did not

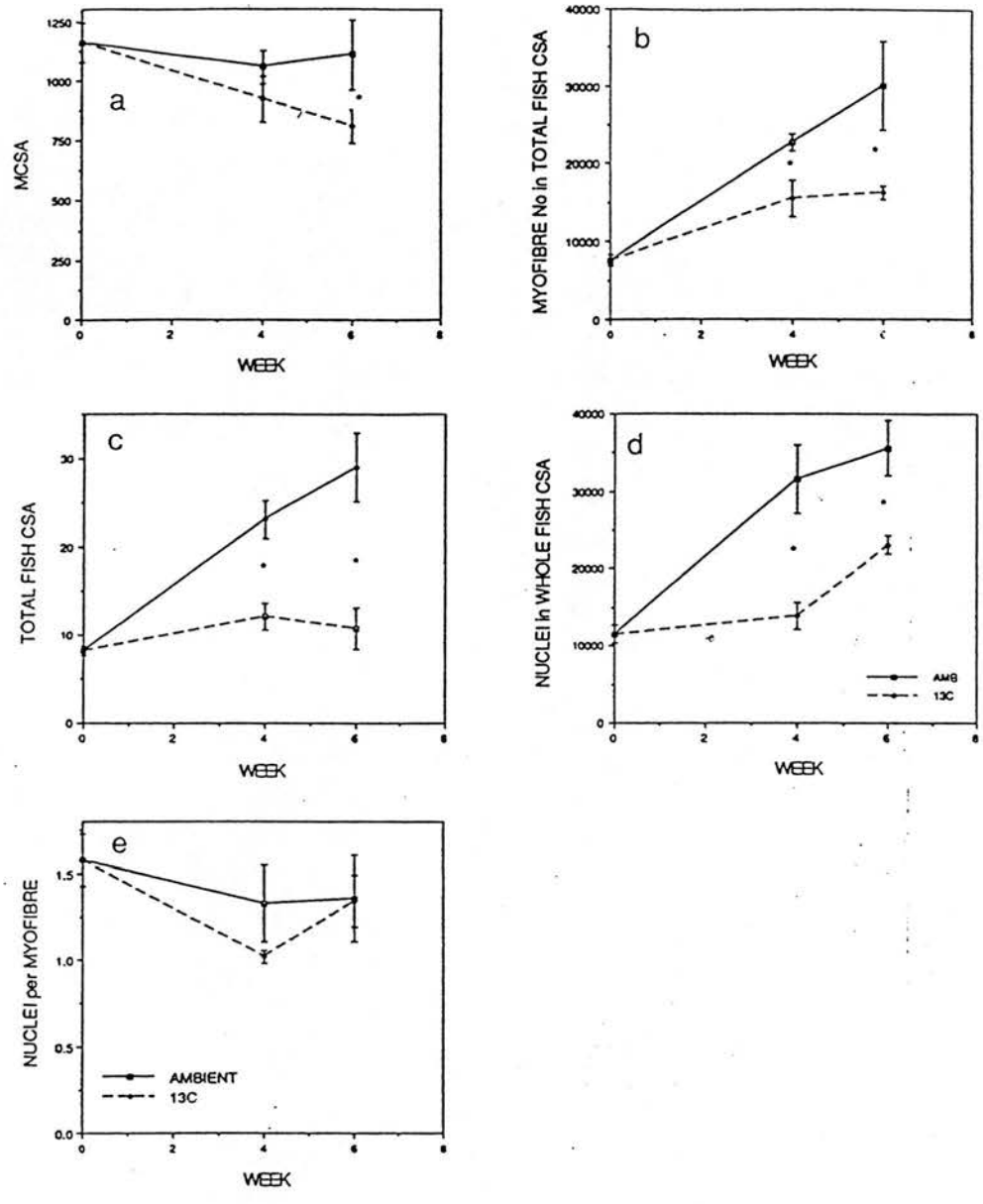

Figure 1 (a) Mean myofibre cross-sectional area, μm^2 (MCSA); (b) total number of myofibres per total fish CSA; (c) total CSA of axial muscle per fish (mm^2); (d) total number of nuclei per whole fish CSA of axial muscle; (e) number of nuclei per myofibre. In sea bass axial muscle of fish reared at 13°C (broken lines) and at 20–26°C (solid lines). An asterisk indicates a significant difference ($P < 0.05$).

Table 1 Change (%) in the number of myofibres per whole axial muscle CSA (Mf No.), the number of nuclei per whole axial muscle CSA (No. of nuclei) and the total axial muscle CSA (total CSA) of fish reared at 13°C and at 20–26°C (AMBIENT) over a period of 6 weeks

Group	Mf. No.	No. of nuclei	Total CSA
13°C	214.6	199.3	120
AMBIENT	369.9	309.2	354

vary between the two temperature groups throughout the period of this study (Fig. 1e).

Discussion

Temperature affects feed intake, spontaneous activity, and growth of fish (Fry 1971; Moksness, Rosenlund & Lie 1995). Sea bass growth can vary remarkably with temperature and this is at least partially due to changes in food intake (Barnabé 1990). In the present study, the swimming and feeding activity of sea bass reared at 13°C was minimal (personal observation). Increased activity and exercise of axial muscle results in increased size and number of myofibres, compared with unexercised or underfed fish (Greer-Walker 1971). In the same manner, the number of white muscle fibres increased more in the ambient temperature group, compared with the fish reared at 13°C (Fig. 1b). It can be concluded that the reduced somatic growth and muscle usage of fish reared at 13°C was associated with reduced white muscle fibre hyperplasia compared with the fish reared at ambient temperature.

A relationship between the temperature-induced differences in metabolism, food intake, somatic growth rate and muscle cellularity has been seen in larval, post-larval and juvenile stages in other fish species (Nathanailides, Lopez-Albors & Stickland 1995a,b; Weatherley, Gill & Rogers 1979, 1980). By the sixth week, there was a significant reduction in the MCSA in the fish reared at 13°C. Protein synthesis and growth of axial muscle increases with food intake (Greer-Walker 1971) and exercise (Houlihan & Laurent 1987). Therefore, the reduction in MCSA of sea bass at 13°C may be due to atrophy of muscle fibres because of the reduced net protein gain in this group. In summary, the increased feeding and swimming activity of the ambient group resulted in increased muscle fibre hyperplasia and somatic growth.

There is some evidence to suggest that in juvenile stages of sea bass, hyperplastic growth of white muscle is associated with the presence of myosatellite cells which provide a source for new myofibres (Veggetti *et al.* 1990). Nuclear proliferation is required for both forms of muscle growth (Cardasis & Cooper 1975). It is known that nuclei in lateral white muscle can be of three types: (a) myofibre nuclei, (b) satellite cell nuclei and a very small proportion of connective tissue or red blood cell nuclei (Alfei, Maggi, Parvopassu, Bertoncello & De Vita 1989). The number of muscle fibre nuclei is important for muscle fibre hypertrophy and muscle satellite cells can provide nuclei for the existing myofibres or differentiate into myotubes (Koumans & Akster 1995). Therefore, a reduction in proliferation of nuclei may be critical for both hypertrophic and hyperplastic muscle fibre growth. The evidence presented in the present study indicates an association of reduced growth with reductions in muscle fibre hyperplasia paralleled by similar reductions in nuclear division.

The relative change of both the total number of nuclei and the total number of myofibres is similar in the two temperature groups (Table 1) suggesting that temperature does not selectively affect the ratio of nuclear division per myofibre or the muscle fibre hyperplasia per body mass. Nevertheless, the fate of newly produced nuclei may vary with temperature. It can be seen that between week 4 and week 6, the number of myofibres did not increase in the 13°C group, whereas the number of nuclei did increase (Fig. 1b, 1d). This suggests that after the fourth week, the proliferation of nuclei from myosatellite cells was used to replenish the population of myosatellite cells (Koumans and Akster 1995) or for supplying nuclei to the existing myofibres.

In conclusion, a temperature induced increase in feeding and swimming activity resulted in increased growth rate. This phenomenon was associated with an increased number of muscle fibres and a parallel increase in nuclei, and there is some evidence to suggest that after 4 weeks at 13°C, muscle fibre hyperplasia is inhibited.

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References

Alfei L., Maggi F., Parvopassu F., Bertoncello G. & De Vita R. (1989) Postlarval muscle growth in fish: a DNA flow cytometric and morphometric analysis. *Basic and Applied Histochemistry* **33**, 147–158.

Barnabé G. (1990) Rearing bass and gilthead bream. In: *Aquaculture*. Vol. 2. (ed. G. Barnabe). pp. 647–696. Ellis Horwood. New York.

Cardasis C.E. & Cooper G.W. (1975) An analysis of nuclear numbers in individual muscle fibres during differentiation and growth: a satellite cell-muscle fiber growth unit. *Journal of Experimental Zoology* **191**, 347–358.

Fry F.E.J. (1971) The effect of environmental factors on the physiology of fish. In: *Fish Physiology*. Vol. VI. (ed. W.S. Hoar & D.J. Randall). pp. 1–98. Academic Press. New York.

Greer-Walker M. (1971) The effects of starvation and exercise on the skeletal muscle fibres of the cod (*Gadus morhua* L.) and coalfish (*Gadus virens* L.) respectively. *Journal du Conseil Internationale pour l'Exploration de la Mer* **33**, 228–244.

Higgins P.J. & Thorpe J.E. (1990) Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon. *Salmo salar* L. *Journal of Fish Biology* **37**, 505–519.

Houlihan D.F. & Laurent P. (1987) Effects of exercise training on the performance, growth, and protein turnover of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fisheries & Aquatic Sciences* **44**, 1614–1621.

Kiessling A., Storebakken T., Asgard T. & Kiessling K.H. (1991) Changes in the structure and function of the epaxial muscle of rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age. I. Growth dynamics. *Aquaculture* **93**, 335–356.

Koumans J.M.T. & Akster H.A. (1995) Myogenic cells in development and growth of fish. *Comparative Biochemistry and Physiology* **110A**, 3–20.

Matshack T.W. & Stickland N.C. (1995) The growth of Atlantic salmon (*Salmo salar* L.) myosatellite cells in culture at two different temperatures. *Experientia* **51**, 260–266.

Moksness E., Rosenlund G. & Lie O. (1995) Effect of fish meal quality on growth of juvenile wolfish. *Anarhichas lupus* L. *Aquaculture Research*. **26**, 109–115.

Nathanailides C., Lopez-Albors O. & Stickland N.C. (1995a) Influence of pre-hatch temperature on the development of muscle cellularity of post-hatch Atlantic Salmon (*Salmo salar*. L.). *Canadian Journal of Fisheries Aquatic Sciences* **52**, 675–680.

Nathanailides C., Lopez-Albors O. & Stickland N.C. (1995b) Temperature and developmentally induced variation in the histochemical profile of mATPase of carp (*Cyprinus carpio*. L.) lateral muscle. *Journal of Fish Biology* **47**, 631–640.

Picket G.D. & Pawson M.G. (1994) *Sea Bass: Biology, Exploitation and Conservation*. Chapman & Hall. London.

Stickland N.C. (1983) Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*). *Journal of Anatomy* **137**, 323–333.

Veggetti A., Mascarello F., Scapolo P.A. & Rowlerson A. (1990) Hyperplastic and hypertrophic growth of lateral muscle in *Dicentrarchus labrax* L. *Anatomy and Embryology* **182**, 1–10

Weatherley A.H. & Gill H.S. (1981) Characteristics of mosaic muscle growth in rainbow trout *Salmo gairdneri*. *Experientia* **37**, 1102–1103.

Weatherley A.H., Gill H.S. & Rogers S.C. (1979) Growth dynamics of muscle fibres, dry weight and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). *Canadian Journal of Zoology* **57**, 2385–2392.

Weatherley A.H., Gill H.S. & Rogers S.C. (1980) The relationship between mosaic muscle fibres and size in rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology* **17**, 603–610.

45. A comparison of light and electron microscope studies on muscle growth in Atlantic Salmon (*Salmo salar*, L.). By A. R. B. BROOKS and N. C. STICKLAND. *Department of Anatomy, The Royal Veterinary College, London*

Muscle growth and development in the majority of vertebrates is by two processes – cellular hyperplasia and cellular hypertrophy. In mammals the full complement of muscle fibres is acquired at or near to birth. Growth after this time is solely by hypertrophy. In fish the muscle mass grows by both hyperplasia and hypertrophy. In Salmonids it has been shown that the two processes occur to varying degrees throughout life.

The light microscope has, in the past, been the main implement in studies on muscle fibre number. Clearly its low resolving power leads to some very small fibres being overlooked. Since the recruitment of new muscle fibres may play a role in the growth spurts of fish it is important to enumerate these very small fibres. Consequently the electron microscope was used in the present study.

Juvenile Atlantic Salmon (*Salmo salar*) were sampled from hatching to the time of seaward migration. Deep and superficial muscle samples were taken from the caudal region of the epaxial lateral musculature. Tissue was frozen for the light microscope, and processed for the transmission electron microscope.

The electron microscope reveals that there is a significantly greater number of very small fibres ($< 50\ \mu m^2$) than that seen using the light microscope. In contrast to what would be expected with respect to muscle fibre orientation the superficial muscle fibres were significantly larger (about 25%) than those found in the deep portion of the muscle. These findings are discussed in terms of fish growth and validity of the light microscope in studies on muscle growth.

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J. Anat. 167, 254

11. The role of temperature in muscle development in Atlantic salmon (*Salmo salar* L.) embryos. By M. L. USHER and N. C. STICKLAND. *Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London*

It has long been recognised that temperature has a direct influence on the rate of embryonic development in fish. A previous study (Stickland. *et al. Anat. Embryol.* **178**, 1988) suggested that temperature exerts a differential effect on hyperplasia and hypertrophy during the development of white muscle in Atlantic salmon (*Salmo salar* L.). The purpose of this study was to investigate the effects of temperature on fibre hyperplasia and myonuclear proliferation in the white muscle of *m. lateralis* during embryogenesis in Atlantic salmon (*Salmo salar* L.).

Three groups of Atlantic salmon eggs (*Salmo salar* L.) were maintained at 5 °C, 8 °C and 11 °C from fertilisation to hatching. Embryos from each group were killed at Gorodilov stages 25, 27, 29, 31 and 33 (hatching), fixed in 1% glutaraldehyde in 0·1 M sodium cacodylate buffer, followed by post-fixation with 1% osmium tetroxide, dehydration in acetones and embedding in Araldite. The cross sectional area of white muscle, total number of white fibres per cross section and myonuclear number were estimated from semithin transverse sections cut from the 10–15 somite region of the embryo.

Cross sectional areas, white muscle fibre number and myonuclear number increased with developmental stage in the embryos of all three groups. At hatching (stage 33), the cross sectional areas of white muscle in the three groups of embryos were not significantly different from each other. However, at hatching embryos reared at 11 °C had significantly fewer fibres than embryos reared at 8 °C ($P < 0{\cdot}01$), which in turn had fewer fibres than embryos reared at 5 °C ($P < 0{\cdot}01$). The numbers of myonuclei per cross sectional area in each of the groups were significantly different from each other ($P < 0{\cdot}05$), being greatest at the lowest temperature and least at the highest temperature. However, embryos reared at 11 °C had a significantly larger number of nuclei per fibre compared to that of the other two groups ($P < 0{\cdot}05$).

Temperature appeared to have a differential effect on the development of muscle with higher temperatures favouring the production of fewer but larger fibres. Given that the mechanism of fibre hyperplasia in developing salmon embryos is far from clear, these results suggested that protein synthesis rather than nuclear proliferation is favoured by higher rearing temperatures.

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Effect of temperature on muscle protein mRNA levels in embryonic salmon (*Salmo salar* L.)

T. W. Matschak, S. Ennion, G. Goldspink and N. Stickland
Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London, Royal College Street, London NW1 0TU, UK

The rate of embryonic development is dependent on temperature in various species (Krogh (1914) *Allg. Physiol.* **16,** 163–77). This holds true also for muscle development in Atlantic salmon. Stickland *et al.* (*Anat. Embryol.* (1988) **178,** 253–7) demonstrated a marked difference in cellularity in salmon embryos reared at 5° and 10°C, respectively. At 10°C white muscle showed an increase in fibre hypertrophy and a reduced rate of hyperplasia compared with fish raised at 5°C. In this study the mRNA levels for muscle protein (actin and myosin) were determined in salmon embryos reared at 5°, 8° and 11°C. Samples were taken at Gorodilov stages 25, 27, 29, 31 and 33 (hatching) (Gorodilov (1983) *GosNIORKh.* **200,** 107–26) and total mRNA was isolated from the 'body' of the embryos, which consists almost entirely of muscle as head and yolk sac had been removed. This RNA was analysed by means of Northern blotting and slot blotting. Probes for actin and myosin derived from a genomic carp library (Gerlach *et al.* (1990) *Am. J. Physiol.* **28,** R237–44) were used. The specificity of the probes was checked on Northern blots and the levels of specific message semi-quantified using slot blots. To standardize the measurements total mRNA levels were determined by means of hybridization with oligo-dT. The actin and myosin mRNA levels were found to be dependent on the developmental stage and the incubation temperature. Both muscle proteins exhibited a similar pattern. At hatching the levels were found to be inversely related to the incubation temperature, i.e. lowest at 11°C, intermediate at 8°C and highest at 5°C. At 5°C and 8°C an increase of the muscle protein mRNA (mpmRNA) levels during development was found. However, at 11°C mpmRNA levels were higher at stage 27 than at hatching. These results were found for myosin and actin mRNAs whether based on total message or on total RNA. It therefore seems that at the higher temperature the transcription of muscle protein is switched on at an earlier age than at the lower temperature. The levels of muscle protein mRNA are a product of transcription and degradation. However, as higher temperatures increase the rate of protein production, it is evident that the mRNA levels are not a direct reflection of protein synthesis. Other regulatory mechanisms such as RNA stability or translation rate must play a role. The high amount of mpmRNA found at 11°C may support the shift to hypertrophy, and therefore protein accumulation, found by Stickland *et al.* (1988, as above) in salmon raised at an increased temperature.

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The growth response of the lateral musculature of carp (*Cyprinus carpio* L.) to temperature. By C. NATHANAILIDES, O. LOPEZ-ALBORS* and N. C. STICKLAND. *Department of Veterinary Basic Sciences, the Royal Veterinary College, University of London and *Department of Anatomy and Embryology, University of Murcia, Spain*

Carp grow faster at warmer temperatures (up to an optimum of 27 °C) than colder temperatures. This investigation studied the effect of temperature on carp muscle in terms of muscle fibre hyperplasia and hypertrophy and in terms of the histochemical profile of constituent muscle fibres.

Young fish (from 1 wk after hatching) and older fish (approximately 6 months) were grown for 5–7 wk at cold (12–17 °C) and warm (25–27 °C) water temperatures. The young fish were killed at 4, 5 and 6 wk after hatching and the older fish at the end of 7 wk temperature acclimatisation. Frozen transverse sections of the lateral musculature were taken just caudal to the vent and were stained for myosin ATPase activity using the method of Mascarello et al. (*Histochemistry* **84**, 1986). At least 150 fibres in a given area were measured and histograms plotted of their size distributions.

From 5 wk onwards there were up to 10 times more fibres in the smallest fibre area category in the warm-acclimated fish than in the cold fish. Very little histochemical differentiation was seen in the youngest fish, but in the 6-wk and older fish the warm acclimatised group exhibited 3 levels of ATPase activity in the white muscle. The darkest fibres were those in the smallest size class. In the cold-acclimatised fish only occasional small, dark fibres could be seen, with all other fibres unstained.

If it is assumed that very small fibres are newly formed, it can be concluded that the warm fish exhibited a greater rate of fibre hyperplasia. Furthermore, it is suggested that the positive myosin ATPase reaction of the small, new fibres may represent an immature isoform of white muscle myosin not found in mature white fibres.

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Does the chorion have an effect on the development of muscle cellularity in pre-hatch atlantic salmon (*Salmo salar* l.)?

T. W. Matschak[1], N. C. Stickland[1] and J. E. Thorpe[2]
[1]Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London and [2]Freshwater Fisheries Laboratory, Pitlochry, UK

Muscle development in embryonic salmon is dependent on temperature, and embryos reared at increased temperatures show an increase in muscle fibre hypertrophy and a reduced rate of hyperplasia from Gorodilov stage 25 (*GosNIORKh* **22**, 107–26, 1983) when compared with fish raised at ambient river temperature (Stickland *et al.*, *Anat. Embryol.* **178**, 253–77, 1988). In contrast, faster growing juvenile and adult salmon show an increase in muscle fibre hyperplasia. During embryonic growth until first feeding the fish are fully dependent on the nutrient supply of their yolk and growth during this period is an important factor for later survival. Additionally, protein synthesis at the expense of nuclear proliferation may represent a more efficient way to acquire an appropriate size. In mammals, for example, energy restriction while maintaining an appropriate protein supply leads to a reduction in nuclear proliferation in muscle with the fibre size remaining uninfluenced (Cheek & Hill, *Fed. Proc.* **29**, 1503–9, 1970). One factor distinguishing the embryos from older fish is the restriction of physical space within the chorion. We therefore removed the chorion from embryos reared at ambient river temperature at Gorodilov stage 25 and incubated the fish in physiological saline at 5°C and 11°C until stage 31 or stage 33. It was found that by stage 31 and, more pronouncedly, by stage 33 the salmon exhibited a statistically significant reduction in average fibre size at the elevated temperature. The total muscle area was also significantly reduced. There was no difference in total muscle fibre number at stage 31 as determined from fibre numbers per unit area. However, at stage 33 the higher temperature fish exhibited a larger white muscle fibre number than their 5°C counterparts. These findings are very much in contrast to the situation found in chorionated salmon embryos. It therefore seems that the absence of a chorion radically alters the effect of temperature on the development of muscle cellularity. This work was supported by the NERC.

29 Effect of prehatch temperature on the posthatch growth of muscle in the Atlantic salmon. By C. NATHANAILIDES, O. LOPEZ and N. C. STICKLAND. *Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London*

It is well known that muscle fibre number is fixed by about the time of birth in mammals. Prenatal nutrition has been shown to affect the determination of muscle fibre number in the fetus; a reduction in muscle fibre number can affect postnatal growth performance. In salmon farming, eggs are sometimes reared at elevated temperatures in order to shorten incubation time (from around 100 to 50 d is possible). Previous work has shown that higher incubation temperatures produce newly hatched salmon with fewer but larger muscle fibres and fewer nuclei than salmon reared at colder temperatures. The purpose of this investigation was to study the effect of differing incubation temperatures on posthatch growth and, in particular, on the further development of muscle cellularity. Eggs from a single pairing of Atlantic salmon were incubated at either ambient temperature (fluctuating around 5 2C) or at 11 °C. At hatching the ambient temperature increased and, by first feeding, was fluctuating around 10 °C. From each group, 5 fish were sampled at hatching, first-feeding and at 3 wk after first-feeding. Transverse resin sections were prepared from each fish at the level of the vent for measurement of total muscle fibre number per section and mean fibre size. During the time period under study, the ambient group grew faster (in terms of body weight and length). As for muscle cellularity the number of muscle fibres remained lower in the 11 °C fish but muscle fibre size increased more in the ambient group so that the difference seen at hatching was eliminated. Work elsewhere has shown that salmon grow faster when temperature is increased posthatch rather than when raised at constant temperatures pre- and posthatch. It is suggested that reduced nuclei in the 11 °C fish at hatching may contribute to the relatively reduced fibre hypertrophy in these fish.

Matschak T.W., N.C. Stickland, P.S. Mason & A.R. Crook (London, U.K.) :
MUSCLE DEVELOPMENT IN SALMONIDS AT DIFFFERENT TEMPERATURES AND OXYGEN LEVELS

Temperature influences the proportionate contribution of fibre hyperplasia and fibre hypertrophy to muscle growth in Atlantic salmon embryos. This has consequences for subsequent posthatch growth. The effect of temperature differs depending on whether the embryos are incubated within or without the chorion. On removing the chorion the O_2 availability to the embryo is substantially increased. The effect of different O_2 levels (50%, 100% and 150% air saturation) and different temperatures (5°C and 10°C) on muscle cellularity were therefore investigated. As in previous findings fibre numbers were lower at the higher temperature (by 17%) and temperature had no effect in dechorionated embryos. A reduction in O_2 decreased fibre numbers at 5°C in chorionated embryos (12%) whereas no effect was found in the dechorionated groups. Fibre cross-sectional area appeared to be affected more by the oxygen levels with a reduction in O_2 leading to a reduced fibre cross-sectional area. Contrary to previous findings an increased temperature led to a smaller fibre cross-sectional area. Nuclear numbers in the presumptive white muscle of the embryos were also determined; dechorionation caused an increase in the number of nuclei at both temperatures (5°C:40%; 10°C:70%). Lowering O_2 failed to reduce the number of white muscle nuclei at 5°C. However, at 10°C, O_2 concentration significantly affected nuclear numbers. Higher O_2 led to 46% more nuclei when chorionated, whilst lower O_2 effected a reduction by 26% in dechorionated embryos. O_2 levels therefore clearly affect muscle cellularity in a temperature-dependent way. Furthermore, it is suggested that O_2 availability is a major contributory factor in the previously observed temperature effects on muscle development in salmon embryos.

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Differences in muscle differentiation of Atlantic salmon (*Salmo salar* L.) embryos in response to temperature and oxygen levels at a late developmental stage. By T.W. MATSCHAK and N.C. STICKLAND. *Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London*

Fish muscle increases in size by either muscle fibre hypertrophy, an increase in cross-sectional area of individual fibres, or by fibre hyperplasia, an increase in the number of fibres constituting the muscle. During embryonic development these processes are influenced by the incubation temperature and, at least during late development in Atlantic salmon, by the amount of oxygen available. By the time of hatching a difference in the number and the cross-sectional area of muscle fibres when eggs are reared under different temperature and oxygen regimes. It is also known,for example, that in herring temperature can lead to asynchronous development of certain organs. In fish, as in other animals, it has been found that a sequence of myosin heavy chain isoforms is expressed during development. Additionally, embryonic fish possess a superficial layer of muscle fibres which initially often exhibit a fast character and later change to a slow fibre type. Frozen cross-sections of Atlantic salmon embryos, at a developmental stage just after hatching, which had been subjected to 5°C and 11°C, and normoxia and hyperoxia (200% normoxia) were stained with an antibody against slow myosin heavy chain. The embryos had been kept at the different conditions from stage 25 to stage 32 after Gorodilov (1983, Sb. Nauchn. Tr. GosNIORKh 200:107-126), i.e. for 40 days at 5°C and for 17 days at 11°C. It was found that, while there was no obvious difference between oxygen treatments within each temperature group, the fish subjected to the lower temperature exhibited staining throughout their superficial layer of muscle fibres, whereas in the high temperature group staining only occurred in the area near the lateral line and at the epaxial and hypaxial extremes. This suggests that, whilst at 5°C all fibres of the superficial layer had switched to the slow type, this was only the case in certain areas at 11°C. It is also known that during early muscle development new muscle fibres are added to the inner muscle fibres in 'growth zones' located towards the surface of the fish and at the epaxial and hypaxial extremes. Later in development new fibres are added throughout the muscle. Serial sections of the above experimental groups were therefore stained with an antibody against proliferative cell nuclear antigen (PCNA), a nuclear proliferation marker. Again no obvious difference was found between oxygen treatments, but in the 5°C group staining was observed throughout the muscle whereas in the 11°C embryos staining occurred only in areas corresponding to the above-mentioned growth zones. The muscle in the 11°C group of fish therefore appeared to be less mature than that in the 5°C group at the time of hatching. This work was supported by the NERC.